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Epigenetic Mechanisms and Regulatory Aspects of Human Cancer and Neural Progenitor Cells: Implications for Diagnosis and Therapy

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Try not to become a man of success but a man of value.

Albert Einstein

I dedicate this thesis
to
*my **parents** and my beloved son **Mahian***

for their constant support and unconditional love.

I love you all dearly.

ABSTRACT

Epigenetic is defined as heritable modification or properties of the functional genome that are not directly embedded in the DNA sequence. Epigenetic changes include DNA methylation, histone modifications and RNA-mediated gene silencing. Genomic imprinting is a mechanism of allele-specific epigenetic control of gene expression according to parent of origin. Alteration of genomic imprinting is associated with tumorigenesis. This thesis is aimed to study epigenetic aspects of human cancer like adrenocortical carcinoma, human cancer cell line, Hep3B and JEG-3 and neural progenitor cells.

In **study I** we have investigated the effect of histone deacetylase inhibitor (HDACi), 4-PB on the gap junctional communication between neuronal progenitor cells (NPC) and glioblastoma multiforme (GBM) cells involving connexin 43 (Cx43). 4-PB increased Cx43 in-vitro, and NPCs may be used as a delivery vehicle for enzyme prodrug therapy for GBM.

The purpose of **study II** was to explore the regulation of neural stem cell differentiation by 4-PB involving *de novo* DNA methyltransferase DNMT3B. For this study we used human embryonic stem cells, NGC-407. Treatment of the human embryonic stem cells, NGC-407 with 4-PB, suppressed astrocytic and neuronal differentiation along with DNMT3B immunoreactivity. This suggests that 4-PB preserve the immature phenotype of NGC-407 by involving epigenetic mechanism of *de novo* methylation.

In **study III** we examined the role of transcription factor PLAG1 on *IGF2* P3 promoter regulation in human cancer cell lines Hep3B and JEG-3. We also studied the ability of PLAG1 to override the insulator effect of the *H19* Imprinting Control Region (ICR) in a GFP reporter construct. We conclude that PLAG1 binding to the *IGF2* P3 promoter is cell line specific and PLAG1 acts as a transcriptional facilitator to partially override the insulation at *H19* ICR.

In **study IV** we identified associations of proteomic profiles with aberration of *H19/IGF2* locus in adrenocortical malignancy. Proteomic profiles were acquired from ACAs and ACCs. We revealed that *IGF2,* along with *miR-483-3P* and *miR-483-5P* overexpression in ACCs, is associated with tumor-type specific proteome profiles. We have shown a positive correlation between *IGF2* and its *miR-483-3P* and *miR-483-5P* along with *H19* and its *miR-675*. We suggest that specific proteomes associated with microRNAs in the *H19/IGF2* locus in ACCs may be used as therapeutic markers and patient follow-up.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to throughout the text by their Roman numerals.

I. Khan Z, **Akhtar M**, Asklund T, Juliusson B, Almqvist PM, Ekström TJ. HDAC inhibition amplifies gap junction communication in neural progenitors: potential for cell-mediated enzyme prodrug therapy. Exp Cell Res. 2007 Aug 1; 313(13):2958-67.

II. Khan Z, **Akhtar M**, Ekström TJ. HDAC inhibitor 4-phenylbutyrate preserves immature phenotype of human embryonic midbrain stem cells: implications for the involvement of DNA methyltransferase. Int J Mol Med. 2011 Dec; 28(6):977-83.

III. **Akhtar M**, Holmgren C, Göndör A, Vesterlund M, Kanduri C, Larsson C, Ekström TJ. Cell type and context-specific function of PLAG1 for *IGF2* P3 promoter activity. Int J Oncol. 2012 Dec; 41(6):1959-66.

IV. **Akhtar M***, Eriksson H*, Caramuta S, Kjellman M, Almgren M, Höög A, Zedenius J, Lui WO, Ekström TJ, Larsson C. Proteome profiles associated with alterations of the *IGF2*/*H19* locus and malignancy in adrenocortical tumors. Submitted for publication.

* Equal contribution

LIST OF ABBREVIATIONS

WB	Western blot
ICC	Immunocytochemistry
ChIP	Chromatin Immunoprecipitation
nChIP	Native Chromatin Immunoprecipitation
IGFII	Insulin Like Growth Factor 2 (Protein)
IGF2	Insulin Like Growth Factor 2 (human gene)
HDAC	Histone Deacetylase
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
NPC	Neural Progenitor Cell
NSC	Neural Stem Cell
GBM	Glioblastoma Multiforme
LUMA	Luminometric Methylation Assay
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
PLAG1	Pleomorphic Adenoma Like Gene 1 (gene)
PLAG1	Pleomorphic Adenoma Like Gene 1 (protein)
LOI	Loss of Imprinting
HOTS	H19 Opposite Tumor Suppressor (gene)
HOTS	H19 Opposite Tumor Suppressor (protein)
NHSS I	Nuclease hypersensitive site I
NHSS II	Nuclease hypersensitive site II
TSA	Trichostatin A
SNP	Single nucleotide polymorphism
LIFR	Leukemia Inhibitor Factor Receptor
PB	Phenyl Butyrate
HAT	Histone acetyltransferase
miRNA	MicroRNA
miR	MicroRNA

BWS	Beckwith-Wiedemann syndrome
ICR	Imprinting control reason
Hep3B	Hepatocellular carcinoma
JEG-3	Choriocarcinoma
ES	embryonic stem cells
5-Aza CR	5-azacytidine
5-Aza- CdR	5-aza-2-deoxycytidine
FDA	Food and Drug Administration
SNP	Single nucleotide polymorphism
gDNA	Genomic DNA
SRS	Silver-Russel syndrome (SRS)
UPD	Uniparental disomy
CTCF	CCCTC- binding factor
LOI	Loss of imprinting
CDKNIC	Cyclin dependent kinase inhibitor $p57^{kip2}$
SAC	Spindle assembly checkpoint
Pol II	Polymerase II
CTNNB1	β-catenin 1
RISC	RNA-induced silencing complex
CLL	Chronic Lymphocytic Leukemia
UTR	Un-translated region
Rb	Retinoblastoma (tumor suppressor protein)
CLF-1	Cytokine-like factor-1
BPGF-1	Bone derived growth factor 1
CGB	Choriogonadotropin beta chain
VEGF	Vascular Endothelial Growth Factor
PIGF/PGF	Placental Growth Factor
SDA	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
RELP	Restriction fragment length polymorphism
PVDF	Polyvinylidene difluoride
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein

Contents

1. INTRODUCTION

1.1 EPIGENETICS

1.1.1 Epigenetic principles

Epigenetics refers to modification or properties of the functional genome that are not directly involved in the DNA sequence but heritable through cell division[1-3]. Or, simply, "epigenetics" is the alterations that are conveyed over several rounds of cell divisions, but that do not change the nucleotide sequence of DNA. In 1942 the term" epigenetic" was first invented by Conrad Waddington to refer to "the interactions of genes with their environment that bring the phenotype into being" [4]. The significance of epigenetic principles is focused by the fact that in every given organism all cells share an identical genome, but they exhibit different functional and morphological properties. The biological events that are not directly programmed in the DNA sequence have been reflected as epigenetic phenomena which include genomic imprinting, differentiation and X-chromosome inactivation. Not only normal biological occurrences, deregulation of epigenetic parameter leads to initiation and progression of disease, notably cancer [2]. Epigenetic footmark is also perceived in several other diseases such as cardiovascular disease [5], type-2 diabetes [6] and developmental syndromes like Prader-Willi, Angelman syndromes [1,7].

1.1.2 Epigenetic modification

Epigenetic changes include DNA methylation, histone modifications and microRNA (miRNA) mediated gene silencing, all of which are vital mechanism known to regulate gene expression[7,8]. There is a close relationship between histone acetylation and DNA methylation and thereby chromosomal remodeling as shown in the Figure 1 . When CpG island in a gene promoter becomes hyper methylated this coincides with deacetylation of local histones. Alternatively hyper acetylation of histone predisposes to methylation of target DNA. [9]

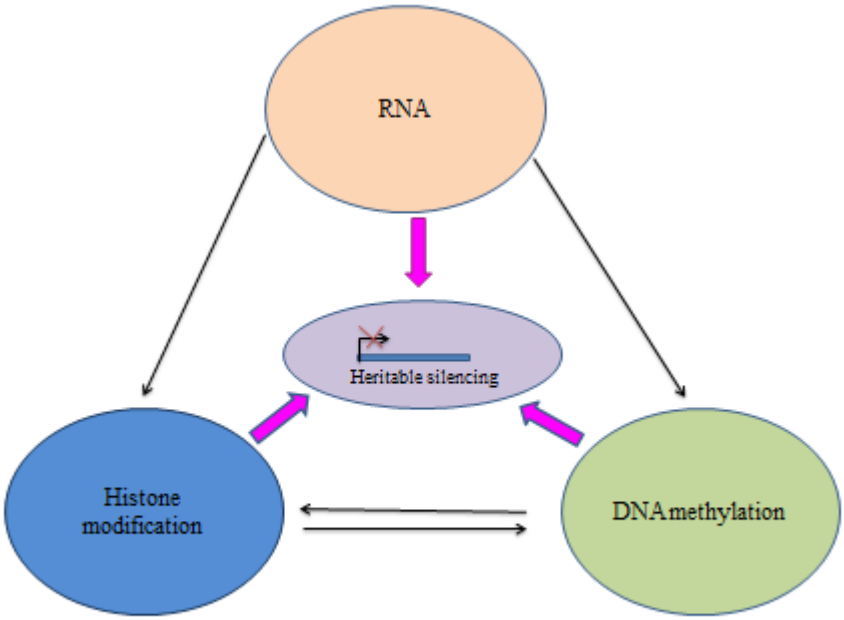

Figure 1. Heritable gene silencing due to interaction between RNA, DNA methylation and histone modification. Histone modification can also initiates CpG methylation by attracting DNA methyltransferase, which in turn can induce histone modification pattern involved in gene silencing as shown by double arrow.

1.1.2.1 DNA methylation

The best studied epigenetic alteration is DNA methylation. The mechanism of methylation is transfer of a methyl group from S-adenosylmethionine to the 5-carbon position of cytosine by an enzyme known as DNA methyltransferase (DNMTs) as shown in Figure 2 below. DNA methylation has important roles in gene expression by enrolling methyl CpG- binding protein. For example, DNA hyper methylation of tumor suppressor genes and recruitment of repressor complexes lead to chromatin condensation and thereby inactivation of gene [3]. Hypo methylation of DNA is also a common event in cancer. Hypo methylation leads to activation of oncogenes or genomic instability which is also frequently observed in cancer [1]. So far in human cancer, two sorts of DNA methylation have been studied: one is global hypo methylation due to overall loss of 5-methyl-cytosine and the other is promoter specific hyper methylation (CpG island specific) [1,2].

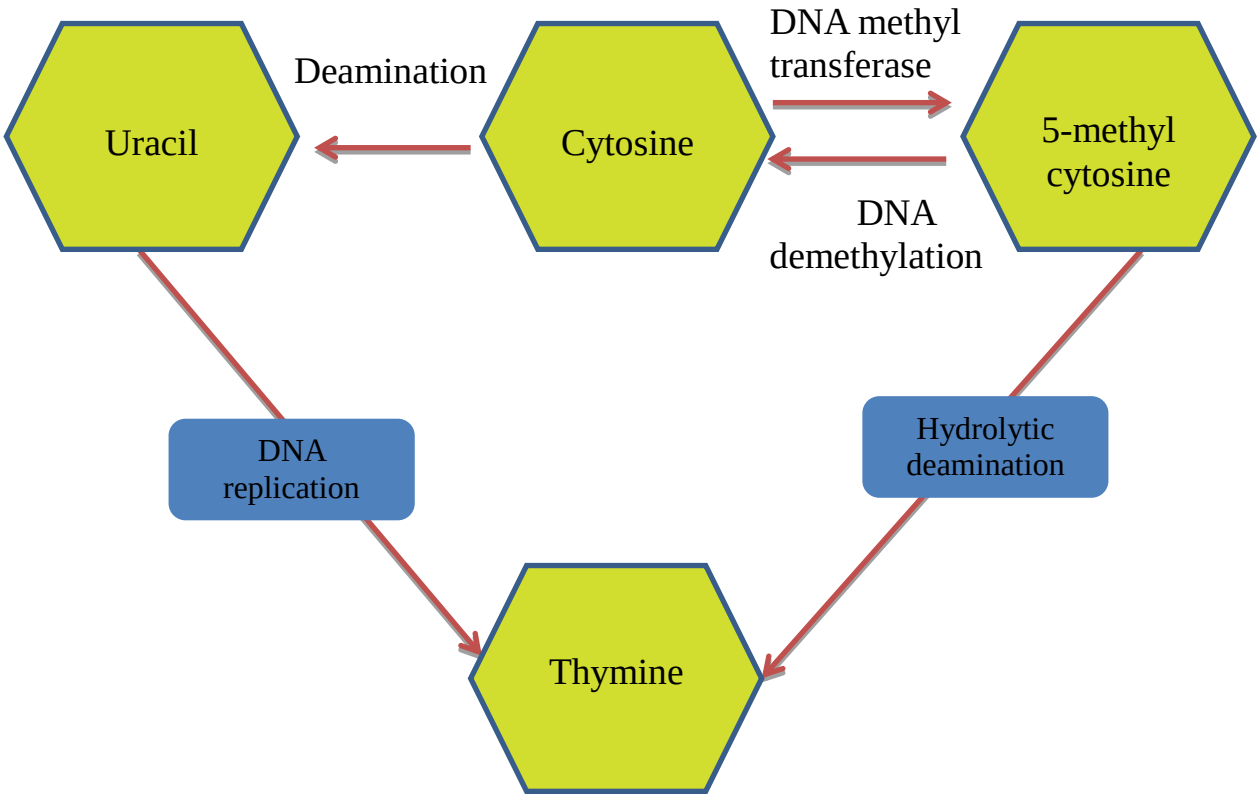

Figure 2. Diagram showing DNA methylation as a reversible procedure and the pathway of cytosine methylation/demethylation. Both methylated and unmethylated cytosine can be converted into thymine and thereby affecting mutation (C to T transition) that contributes to under-representation of cytosine in the genome.

1.1.2.2 Histone modification

Another important event in cancer is histone modification which is characterized by covalent alteration of N-terminal tail of core histones (H2A, H2B, H3 and H4). Most studied histone modification in cancer is acetylation. The enzyme responsible for histone acetylation is histone acetyltransferase (HATs) which acetylates lysine and arginine residues of histone tails leadings to an open chromatin structure so that transcription regulator can have access to promoter region and gene activation [10]. Histone Deacetylase is another class of enzyme which takes away the histone acetyl groups and thereby prevents transcription as shown in Figure 3. Other histone modifications are methylation, phosphorylation, biotinylation and ubiquitination [11-13]. Histone modification has a major role in initiation of cancer as well as progression of malignancy [14].

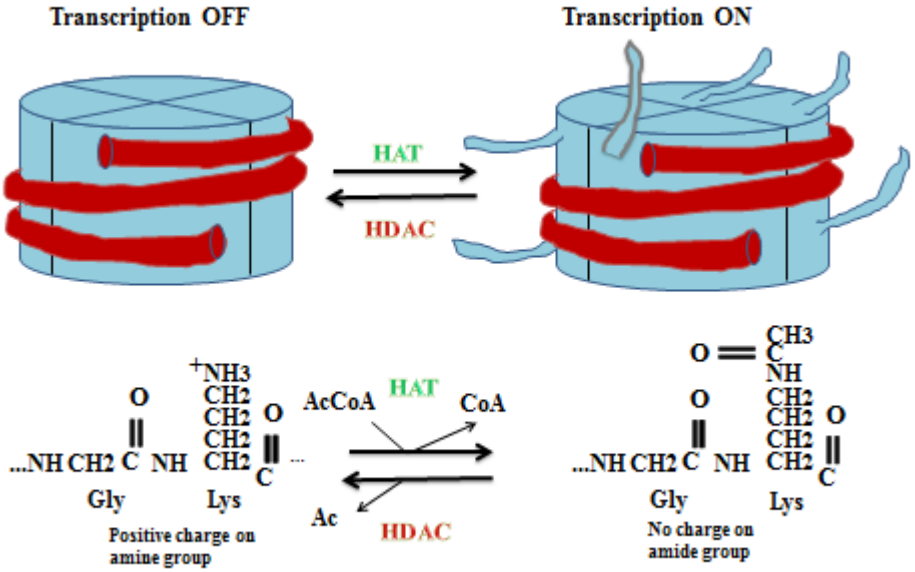

Figure 3. Role of histone acetyltransferase and histone deacetylase on activation and silencing of a gene, respectively. HAT adds acetyl to lysine residue and thereby takes away positive charge form the amino group and HDAC reverses the state.

1.1.2.3 MicroRNAs (miRNAs or, miR)

MicroRNAs are 20 to 30 nucleotide long non coding RNAs which has negative regulatory effect on target genes [15]. After formation of mature miRNA, it is integrated into a compound known as RNA-induced silencing (RISC complex). RISC-miR complex can target mature RNA as shown in Figure 4. The miRNAs targets CpG island of a promoter area, binds to the 3` untranslated region of target mRNA specially a number of protein coding transcript and thereby alters DNA and histone methylation or degradation of mRNAs which leads to post transcriptional down regulation of target gene [16-18] as shown in Figure 4. In a typical cell miRs are strongly regulated whereas deregulation of miRs leads to cancer [19,20]. Not only in carcinogenesis, several studies have shown over expression of miRs as a basis for progression and metastasis of cancer as shown in Figure 5, for example when miR-21 is over expressed in breast cancer can stimulates metastasis into lung [21,22]. A number of studies have revealed that miRs can be used as diagnostic and prognostic markers [23].

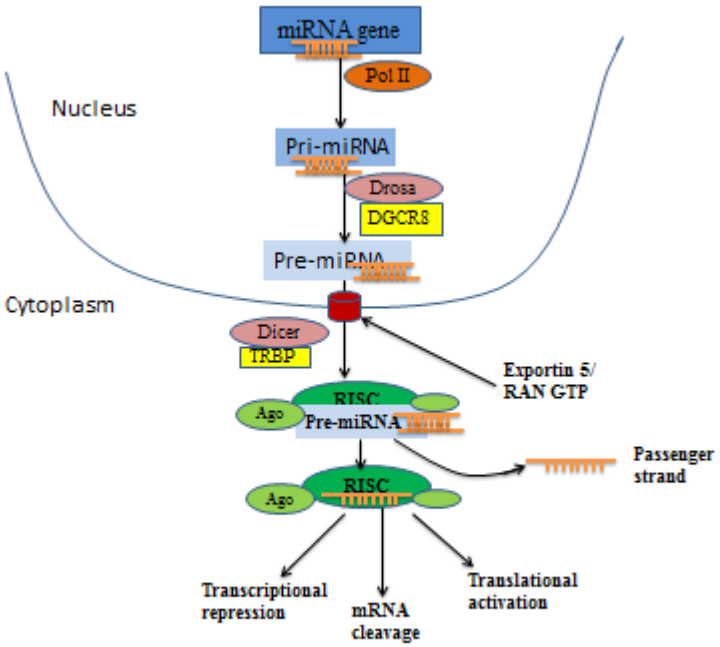

Figure 4. Biogenesis and mechanism of action of miRNAs

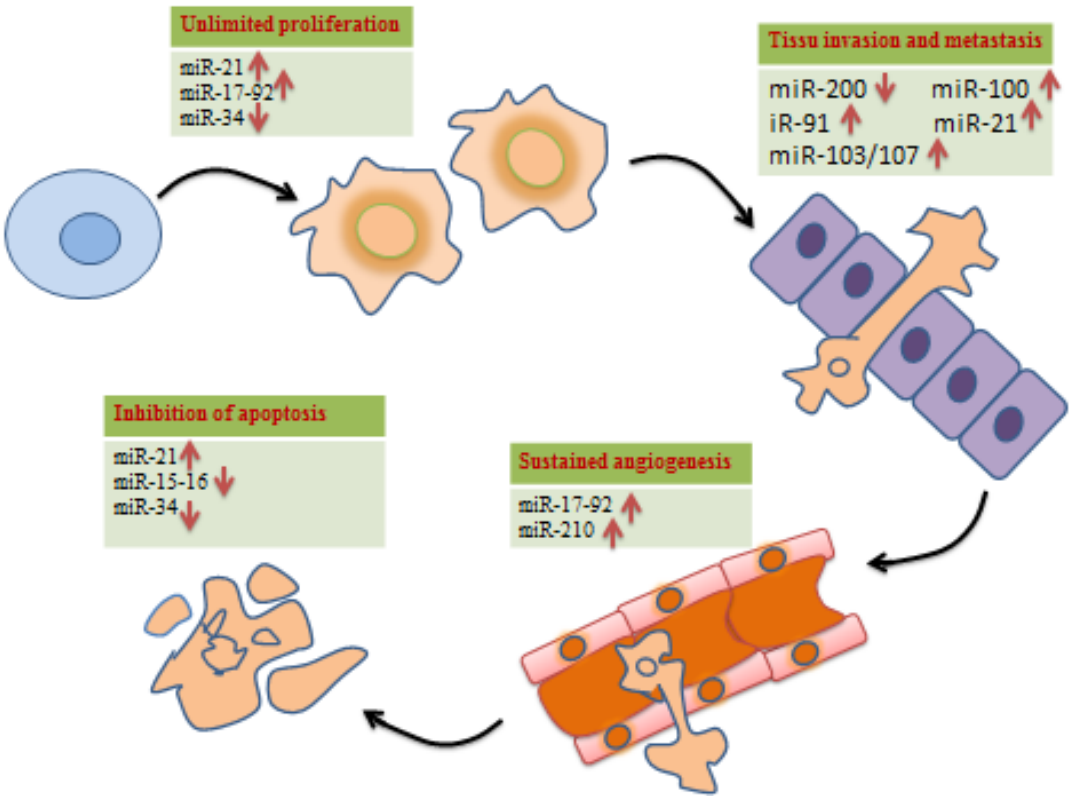

Figure 5. Function of deregulated miRNAs which may either re-expressed tumor suppressor gene or activate oncogene to cause excessive cellular proliferation, metastasis, and angiogenesis and inhibits apoptosis.

1.2 EPIGENETIC PROGRAMMING DURING CELLULAR DIFFERENTIATION

During cellular differentiation certain genes need to be activated and while others need to be silenced to make correct cell lineage decision. Establishment of precise gene expression pattern is synchronized by epigenetic mechanism including histone acetylation and DNA methylation.

1.2.1 Role of Histone Deacetylases in differentiation

Genes involved in cellular differentiation processes in various tissues are regulated by Histone Deacetylases (HDACs) shown in Figure 6. Many studies have shown that HDACs inhibitors (HDACis) can modulate the differentiation of neuronal, epithelial, adipocyte, osteogenic, muscle cells and hematopoietic cells and in most of the cases HDACi induces terminal differentiation [24].

In cancer, stability between proliferation and differentiation is disrupted: tumor cells often keep an immature phenotype by exhibiting altered or no differentiation and by showing limitless proliferation as shown in Figure 6. HDACi may restore the stability by stimulating differentiation of tumor cells and reverse their potential for proliferation. Acute myeloid leukemia in which myeloid differentiation is blocked, is an outstanding disease model to investigate the parameter of differentiation and cancer progression [25]. Not only in hematological malignancy, several studies have shown a role of HDACi in differentiation of glioblastoma cells [26], in breast cancer cells [27] , hematoma cells [28], endometrial stromal sarcoma [29] and small cell lung cancer cells [30].
Most of the studies have been shown that HDACs cause blockage of differentiation as a hallmark of cancer and HDACi reestablishes it. In neuroblastoma a particular HDAC named HDAC8 has been shown as a vital regulator of tumor cell differentiation [31]. Progression of neuroblastoma is associated with high HDAC-8 expression. By knocking down HDAC-8 or treating with HDACi reserves cellular proliferation and stimulates neuronal differentiation, which emphasizes HDAC-8 as a promising target of differentiation therapy [31].

Although little is known about HDACi to specific HDACs, a HDAC8-selective inhibition by siRNA [31] has been shown to induce neuronal differentiation and inhibition

of proliferation in neuroblastoma cells. A study has also shown that HDAC2 knockdown causes apoptosis in the same cell, which suggest that distinct cellular programs are regulated by different HDACs [31].

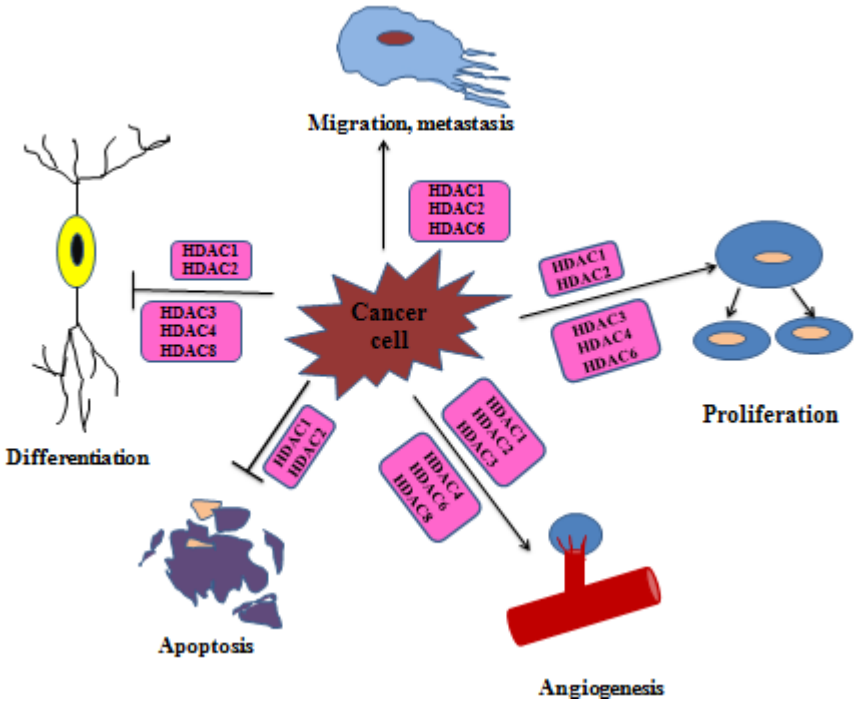

Figure 6. Role of histone deacetylases in cellular differentiation, proliferation, apoptosis and migration/metastasis.

1.2.2 HDAC inhibitors (HDACis) on cellular differentiation

HDACis are novel classes of drugs that function by epigenetic remodeling and thereby lead to changes in expression of target genes which are involved in regulation of cell cycle, apoptosis, proliferation and differentiation. Most often it causes reactivation of certain genes like p^{21} [28,32]. HDACi stimulates Cyclin-dependent kinases (Cdk)/Cyclin complexes which are responsible for Retinoblastoma (Rb) protein phosphorylation and thereby arrest of G1 phase of a cell cycle which ultimately arrest cell cycle at S phase and DNA replication as shown below in Figure 7.

The mechanisms include:

- HDACi causes up regulation of genes transcript that controls G1/S phase progression, most important is Cdk inhibitor CDKN1A (p21) [33-35].

- HDACi at high concentration causes hyper acetylation and thereby G2 phase arrest. Cell lines that are arrested in G2 phase are resistant to the cytotoxic effects of these drugs. If the effect of HDACi on G2 phase arrest is lost, cells go into mitotic phase and become sensitized to apoptosis by these drugs. [36-38]

- When cells enter aberrant mitosis, a system known as spindle assembly checkpoint (SAC), arrest cells into mitosis until defects are repaired. If repair cannot be achieved within > 16 hrs then apoptosis is triggered which indicates a selective cytotoxicity action of this drug. [36,39,40]

HDACi have been tried for the treatment of varied disease like autoimmune disease [41], muscular dystrophies, inflammation [42], immune disease [34], neurodegenerative disease [43] and latest as anti-HIV agents [44].

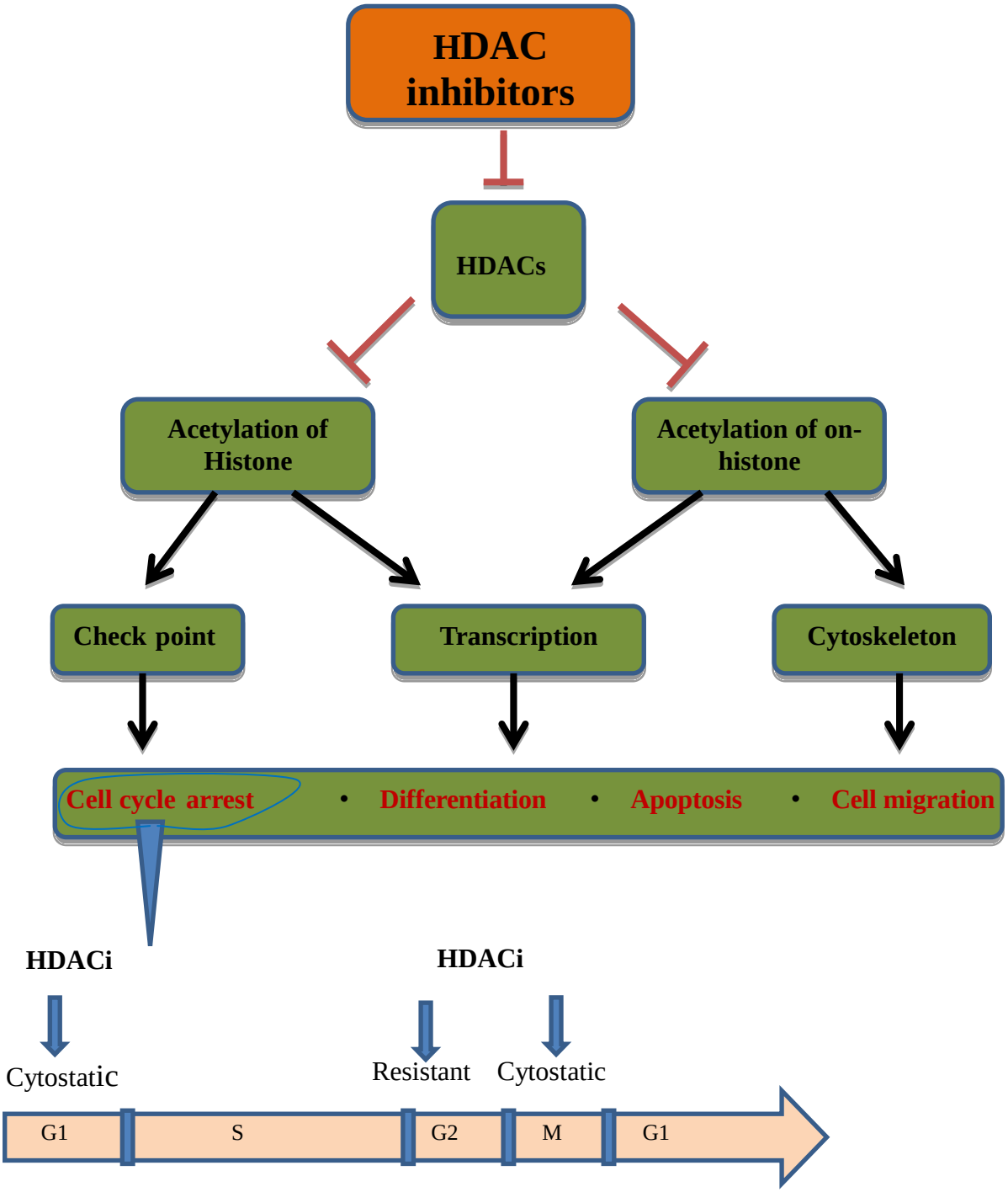

Figure 7. Schematic diagram showing possible mechanism of action of HDACi on cell cycle and differentiation. Treatment with HDACi induces acetylation of histones and non-histone proteins thereby inducing two distinct downstream pathway activations. HDACi at high concentration causes hyper acetylation of histones which modulate expression of subsets of genes responsible for cell cycle arrest, differentiation and apoptosis. Hyper acetylation of histones also induces a G2 cell cycle checkpoint, a phase that is lacking in cancer cells, and a reason of aberrant mitosis in cancer.

1.2.3 DNA methyltransferase and cellular differentiation

DNA methylation is a hallmark of genomic imprinting and regulates gene expression and cellular identity. In human, there are three active DNA methyltransferases (DNMTs) named DNMT1, DNMT3A and DNMT3B. DNMT3A and DNMT3B are *de novo* enzymes associated with establishment of DNA methylation during development while the maintenance DNMT1 is in charge of methylation of newly synthesize DNA strand to preserve the epigenetic regulatory state after cellular replication [45,46]. In addition to these there is another DNMT which is catalytically inactive known as DNMT3L, which stimulates the function of DNMT3A-and -3B and suppress gene expression after association with HDACi [47].

During embryonic development, multipotent and pluripotent cells can differentiate into hundreds of distinct type of cell types and DNA methylation plays a major role to maintain differentiated states by silencing of responsible gene for differentiation.

To preserve stem cell pluripotency, DNMT1 and other DNMTs are not continuously required to suppress differentiation and thus maintain the pluripotency. By regulating cell cycle and DNA repair pathway, DNA methylation maintain cellular differentiated states. Such as in epidermis and hematopoietic tissues, deficiency of DNMT1 prevents cellular proliferation, self-renewal and premature differentiation. Extensive changes in DNA methylation is observed only in progenitor cells of normal epidermal differentiation along with normal expression of DNMT1 whereas differentiated cells do not show such type of methylation changes [48,49]. Knockdown of DNMT1 in undifferentiated epidermal progenitor cells lead to cell cycle arrest, premature differentiation and a failure of tissue self-renewal. Loss of DNMT1 is associated with de-repression of genes responsible for differentiation and loss of cell cycle progression gene which specifies the importance of DNA methylation and DNA binding protein for maintaining of progenitor states and thereby inhibits differentiation [48-50].

DNA methylation also has vital role in regulating cellular multipotency (rapidly self-renewal ability). Such as DNMT1 regulates cell cycle by suppressing p15INK4B and p16 [INK4A]. Deletion of DNMT1 is associated with loss of proliferative stamina which is partially rescued by Cyclin D1 and Cdk4 expression [51,52].

This study has also shown that after addition of Gadd45 A/B in demethylation of DNA causes active cellular proliferation, differentiation and methylation of specific differentiation promoters as shown in Figure 8.

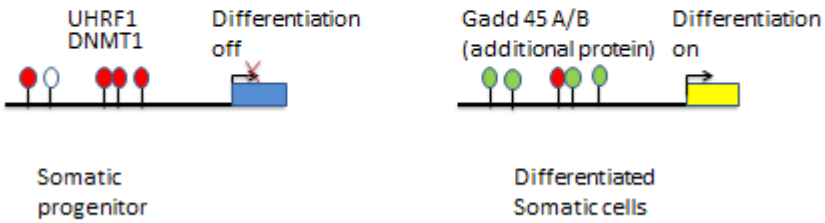

Figure 8. Figure showing role of DNMT1 in regulation of DNA methylation in somatic progenitor differentiation. On left, in un-differentiated progenitor cells, DNMT1 along with its physical binding partner UHRF1suppress expression of differentiation gene, thereby repress differentiation. In the differentiated states (right), DNMT1 is down regulated and mediator of active DNA demethylation specially Gadd45A/B protein promotes DNA demethylation which leads to promoter de-repression and thereby causes induction of differentiation.

1.3 PROSPECT OF EPIGENETIC THERAPY

Epigenetic changes are reversible which makes it different from genetic changes and this is the basis for expected benefits of epigenetic therapy. The object of this treatment principle is to reverse epigenetic changes, especially DNA methylation, histone modification and expression of miRNA target genes, which leads to carcinogenesis, and thereby to reestablish a normal epigenome.

So far two groups of drugs have been reported that target the epigenome: DNA demethylating agent and HDACi. Both of them functions by re-establishing some functions, e.g. tumor suppressor gene expression, often aberrantly silenced in cancer tissue due to epigenetic mechanism.

1.3.1 DNA methylating agent and epigenetic therapy in cancer

Nucleoside analogs are available that inhibit DNMTs. These include 5-azacytidine (5-aza CR) or Vidaza and 5-aza-2` oxycytidine (5-aza- CdR) which is known as Decitabine . The latter has been approved by the Food and Drug Administration (FDA)

for treatment of selected hematological malignancies. Zebularine is another demethylating drug used for cancer [53]. These inhibitors lower DNA methylation by inhibiting DNA methyltransferases and thereby cause re-activation of silent genes [7,54] as shown in Figure 9.

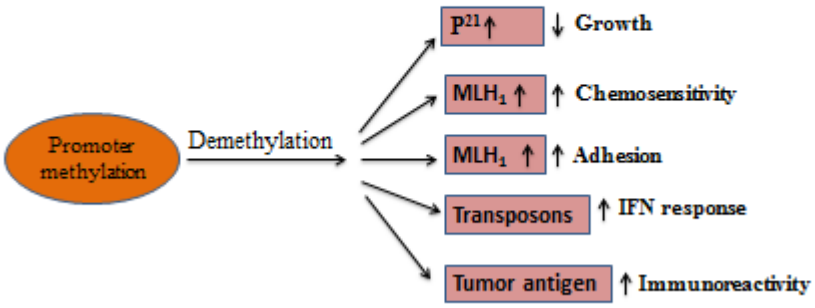

Figure 9. DNMTi reactivates aberrantly silent gene

1.3.2 Histone modification therapy of cancer

HDACi is a class of epigenetic drugs which inhibits the function of HDACs thereby removing acetyl group from histone tails. Valproic acid and the suberoylanilide hydroxamic acid (SAHA) or Vorinostat, are two currently available HDACi which acts by inhibiting class I HDACs (HDAC1-3 and HDAC8) and class II HDACs (HDAC4-7, HDAC9 and HDAC10). [7] These drugs affect cancer cells by increasing expression of differentiation markers, influencing cell cycle and inducing of apoptosis [55-57], with less toxic effect on normal cells [58,59]. Anticancer effects of HDACi are through transcriptional changes. HDACi can arrest cell cycle progression (Figure 10) by blocking at multiple points mainly in G1, G2 and mitosis. At present HDACi is in clinical trial either in combination with other drugs or as monotherapy. For example, Vorinostat is approved for the treatment of cutaneous T-cell lymphoma and is an example of principles for future cancer therapy [60,61].

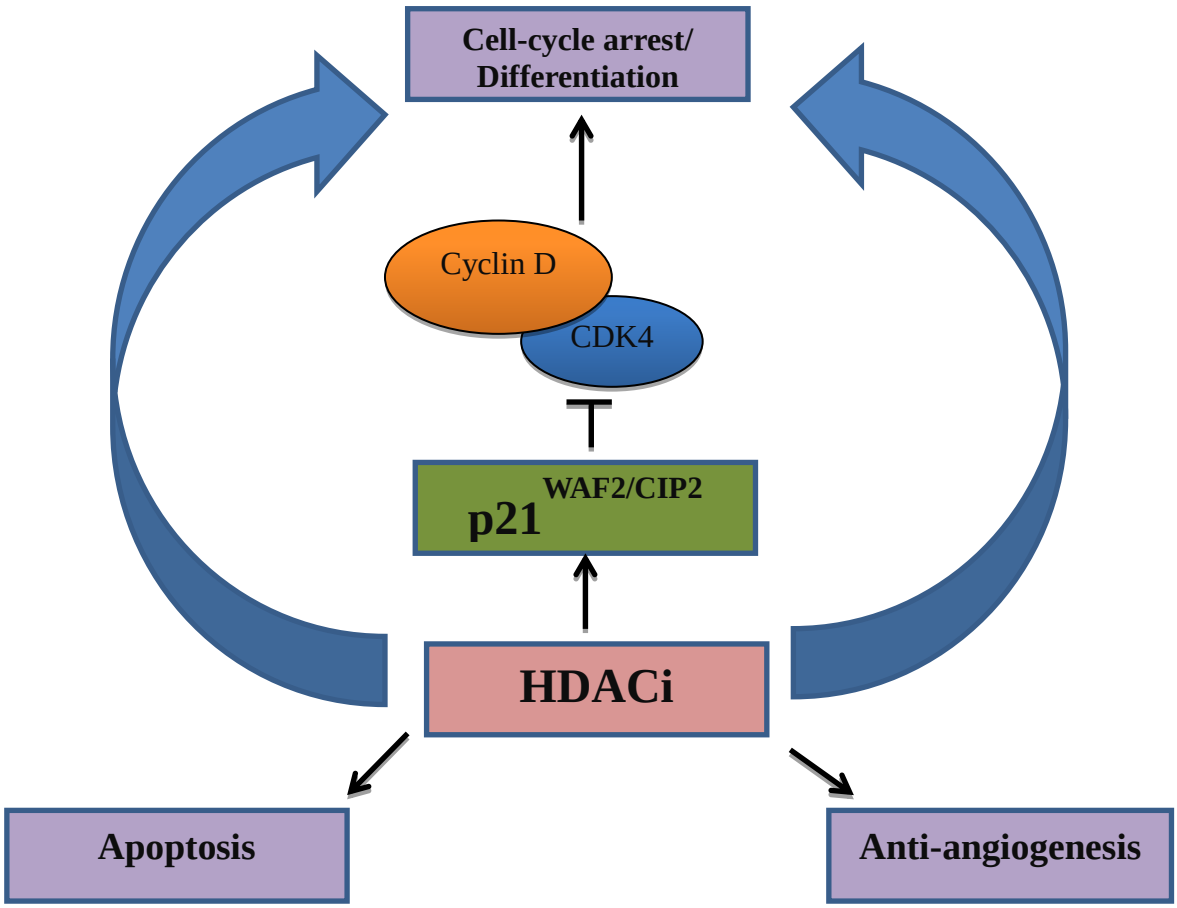

Figure 10. Effects of HDACi in human cancer

1.3.3 MicroRNAs as a therapeutic targets

MicroRNA- centered therapy has been proposed for the treatment of cancer, either alone or combination therapy. Therapeutic aspect of miRNAs is based on its ability to target multiple cellular events such as proliferation, differentiation and survival. MiR-181 has been shown to be a target for T cell receptor sensitivity. MiR 15a/16-1 has been studied for leukaemic MEG01 cell apoptosis and also for inhibition of tumor growth in a xenograft model. So far, two key approaches have been used to target miRNA expression in cancer. One is a direct method in which oligonucleotide or virus vectored miRNA is used to halt the expression of oncogenic miRNA and another is indirect method in which tumor suppressor miRNA that has been lost in cancer, is restored. [23]

1.4 INSULIN LIKE GROWTH FACTOR 2 (*IGF2*)

Key genes of the *IGF2/H19 l*ocus are Insulin like Growth Factor 2 (*IGF2)* and *H19.* In our study we have emphasized on *IGF2* and its role in cancer development. Human *IGF2* gene is located on the short (p) arm of chromosome 11at locus 15.5 and extends 30 kb [62]. The gene product, the IGFII protein, is fully processed, 67 amino acids [63]. *IGF2* is located close to the insulin gene and < 200 kb away from *H19* gene [63-65].

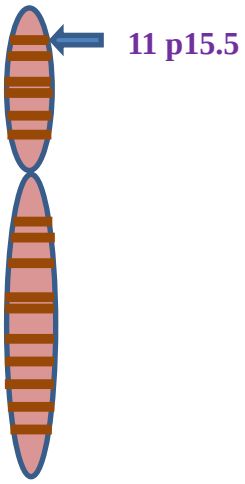

Figure 11. Human chromosome 11showing position of *IGF2*/*H19* locus

The Human *IGF2* gene consists of nine exons and five different promoters numbered as P1, P0, P2 - P4, creating transcripts with common coding sequences at exons 7,8 and part of 9 [66] (Figure 12). The different transcript produced by the *IGF2* gene and the putative transcription factors for the promoters are shown in **table1**.

P1 is the main promoter of *IGF2* transcription in human postnatal and adult liver. Its transcript and transcription factors are shown in Table 1.[67].

The P2 transcript is 5.0 kb [68] and lowly abundant in human fetal and adult tissue with some rise in human tumor tissue [69].

The P3-promoter produces 6.0 and 2.2 kb mRNAs [67] expressed in fetal and mature non hepatic tissues and in most *IGF2* expressing cell lines. This promoter contains a functional TATA box and a CAAT-box. The distal promoter region (-1300/-289) is responsible for cell type specific *IGF2* expression. In Hep 3B and HeLa cells expression occurs from the proximal promoter region [70].

Name	P1	P0	P2	P3	P4
Expression	Post natal and adult liver	Placenta All adult tissues except brain and fetal skeletal muscles	Fetal tissue, some cancer (low) Low amount in some adult tissues.	Fetal tissues and cancer tissues	Fetal tissues and cancer tissues. Low amount in some adult tissues.
Transcript (mRNA)	5,3 kb	5kb	5.0kb	6.0kb (abundant) 2.2 kb (minor)	4.8kb
Transcription factors	Sp1, C/EBPα, C/EBPβ, HNF-3β HNF-4 (repressor) RAR RXR (TATA less promoter)		No transcription factor, Weak promoter without TATA or CAAT less promoter	TBP(TATA binding protein) p53 (repressor) CTF(CAAT transcription factor) Sp1 Egr-1(+++) Egr-2 WT(repressor)	Sp1 is major regulator of P4, Egr-1 RB

Table 1. *IGF2* promoter specific transcripts and its transcription factors

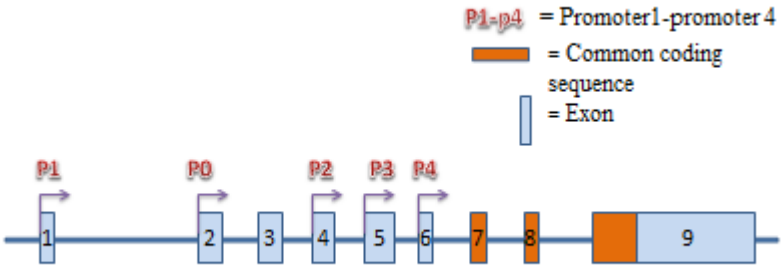

Figure 12. Structural anatomy of human *IGF2* gene. Nine exons are shown by boxes numbered as 1-9. Common coding regions are marked by orange colored boxes. Five promoters are numbered P1, PO, P2-P4 and transcription starts sites are indicated by arrow.

Insulin like growth factors (IGFs) are potent mitogen for cellular growth and differentiation. IGFs that are locally produced, have important role in wound healing and regeneration. In human serum IGFII levels range from 100ng/ml to 300 ng/ml from week 30 to term pregnancy respectively. After birth serum level of IGFII increases up to 700 ng/ml. During embryonic period, *IGF2* transcripts are produced by P2, P3 and P4, among which the P3 producing 6 kb mRNA is the most active one. The activity of these promoters goes down after birth within the first year of life [71] . Subsequently, P1 promoter becomes activated and its 5.3 kb transcript is the main *IGF2* encoding transcript from first year to adult liver.

1.5 GENOMIC IMPRINTING

Genomic imprinting is a biological phenomenon affecting gene expression and which is under epigenetic control, in which one allele is differently expressed depending on parent of origin [72,73]. It has been shown that mouse embryo cannot develop normally if both alleles are either only of paternal or maternal sources. This verifies that to have a complete viable embryo both parental contributions is required [74,75]. Equal parental contribution is also vital for development of the human embryo e.g. hydatidiform molar pregnancy is an androgenous embryo which lacks maternal genome [76,77]. As a result it does not turn into viable fetus. Conversely, ovarian teratoma is grown due to spontaneous duplication of an ovarian oocyte, lacking paternal genome [78]. Studies have shown that mice with balanced translocations containing small genomic regions originating only from the father or mother, is able to develop into mice embryo (UPD). This reveals the restriction of biparental inheritance to certain area of genome [79]. These positions have been shown to harbor clusters of imprinted genes. The first imprinted gene discovered was the murine *Igf2* [80] . In 1993 also the human *IGF2* gene was shown to be imprinted [81]. Genomic imprinting has likely an important role in preventing parthenogenesis and Parthenogenic mice die within 10 days of embryogenesis. The imprinted *IGF2*/H19 locus has key role in this since genetic manipulation of it can allow for a viable mouse fetus with two maternal genomes. [82].

So far 100 imprinted genes have been recognized in human and mouse genomes. Most of the imprinted genes remain in clusters [83] which are rich in CpG islands [84]. Imprinting is erased just after fertilization, reprogrammed and then reset again during gametogenesis [72,83]. In mammals 80% the imprinted genes are located in clusters or closed proximity to each other [72,73]. A gene is termed imprinted when its expression is suppressed due to parent of origin. Interesting examples of two diverse disorders that can progress due to the loss of the same genetic segment of chromosome 15 are Prader-Willi and Angelman syndromes, which arise specifically depending on parent who donates the deletion [85].

1.5.1 The *IGF2/H19* mechanism for genomic imprinting

Imprinted genes are placed in clusters and most of the time they share common imprinting control regions (ICR), also called differentially methylated regions, (DMR). The cluster of imprinted genes located on 11p15.5 locus includes *IGF2, H19, CDKNIC, KCNQ1 and KCNQ1OT1*. The cluster is distributed into two domains. One domain controls expression of imprinted genes *IGF2* and *H19* and located at the end of the telomere and known as the *IGF2/ H19* locus, Figure 13. Another domain is located proximal (towards centromere) to the *IGF2/ H19* locus and controls the imprinting of a number of genes together with *CDKN1C* (Cyclin dependent kinase1), *KCNQ1 (*a voltage gated channel1) and *KCNQ1OT1* (antisense RNA). Disorder of 11p5.5 imprinting region leads to two imprinted clinical phenotype: Beckwith-Wiedemann syndrome (BWS) and Silver-Russel syndrome (SRS). [86,87]

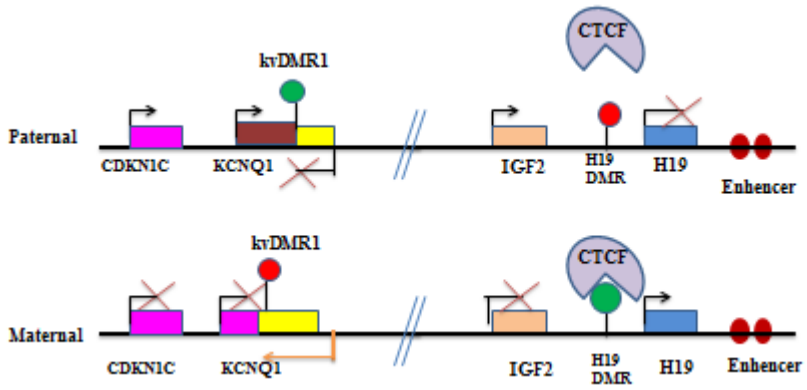

Figure 13. Imprinted gene clusters at *IGF2/H19* locus

IGF2 is a maternally imprinted and *H19* is a paternally imprinted gene located in the *IGF2/H19* locus. At DMRs, methylation of the two parental alleles is different depending on the parent who donated the allele. An important feature of epigenetic marks in DMRs is they are erased during gametogenesis and re-established in primordial germ cells to replicate parental characteristics for the subsequent generation [88,89]. DMRs regulate imprinting by enrolling or inhibiting binding of an insulator at the methylated or un-methylated CpGs in the DMR. The DMR at the *IGF2/H19* locus is one example of this. The *H19* DMR is situated just upstream to *H19* (around 2 kb) and downstream to *IGF2* and this is the site for binding of the zinc-finger protein CCCTC-binding insulator factor CTCF. On the maternal allele, CTCF binds the *H19* DMR when it is un-methylated, thereby insulating the *IGF2* promoter from the *IGF2*/*H19* common enhancer located downstream of *H19* as shown in Figure 13. This makes the

enhancer to interact with *H19* promoter thereby causes expression of *H19* whereas *IGF2* expression is silenced. In contrast, the *H19* DMR on the paternal allele is methylated which prevents CTCF to bind thereby allowing the *IGF2* promoter to interact with the *H19* downstream enhancer and *IGF2* expression. Additionally the methylation of the DMR on the paternal allele is spreading into the *H19* promoter, thereby repressing it. [90,91]

1.5.2 Loss of imprinting of *IGF2,* LOI

Pathogenic modification of DNA methylation without change in the DNA sequence is known as epimutation. Mutation or epimutation can both be involved in imprinting disorders (see e.g. Prader-Willi and Angelman as mentioned above). Common mechanism of imprinting disorder are: (i) Uniparental disomy (UPD), (ii) copy number variation that directly change imprinted gene function and (iii) mutation or epimutation at DMR/ICR that results in biallelic expression of imprinting genes. [83,92,93]. Biallelic expression of *IGF2* is known as loss of imprinting (as shown in Figure 14), LOI of *IGF2* which can be either due to reactivation of already silent maternal allele or duplication of the active paternal allele (UPD).

Indeed BWS and SRS, LOI of *IGF2* is observed in several human cancer e.g. Wilms' tumors [94], colorectal carcinoma [95] and ovarian cancer [96]. Somatic epimutation is the cause of LOI of *IGF2* in both sporadic embroynal tumor e.g. Wilms` tumor or sporadic adult neoplasia like colorectal cancer, chronic myeloid leukemia, ovarian carcinoma, renal cell carcinoma, lung adenocarcinoma, esophageal cancer, meningioma's, hepatoblastoma, osteosarcoma, skeletal muscle tumor and some other cancers [97]. Precancerous conditions like colonic polyps and development of nephrogenic rest in kidney present with high *IGF2* expression due to LOI. This stimulates autocrine and paracrine growth and leads to cell hyperplasia and cellular proliferation [98,99]. In some colorectal cancer patients LOI of *IGF2* has been detected in adjacent normal tissues and also in blood which is suggestive of blood *IGF2* as a biomarker for colorectal cancer [100]. A study has also shown partial LOI of *IGF2* in normal individuals, which is inherited down the generations [101]. Cruz-Correa *et al* has shown that LOI of *IGF2* is not age related but instead it is a stable epigenetic phenomenon [102].

H19 is recognized as a tumor suppressor gene, the transcript of which is a noncoding RNA. *H19* DMR is hypo methylated in bladder cancers [103] and LOI of *H19* with

biallelic expression has been publicized in lung cancer by Kondo *et al*, 1995 (abstract). The *H19* locus also harbors the microRNA, miR-675 [104].

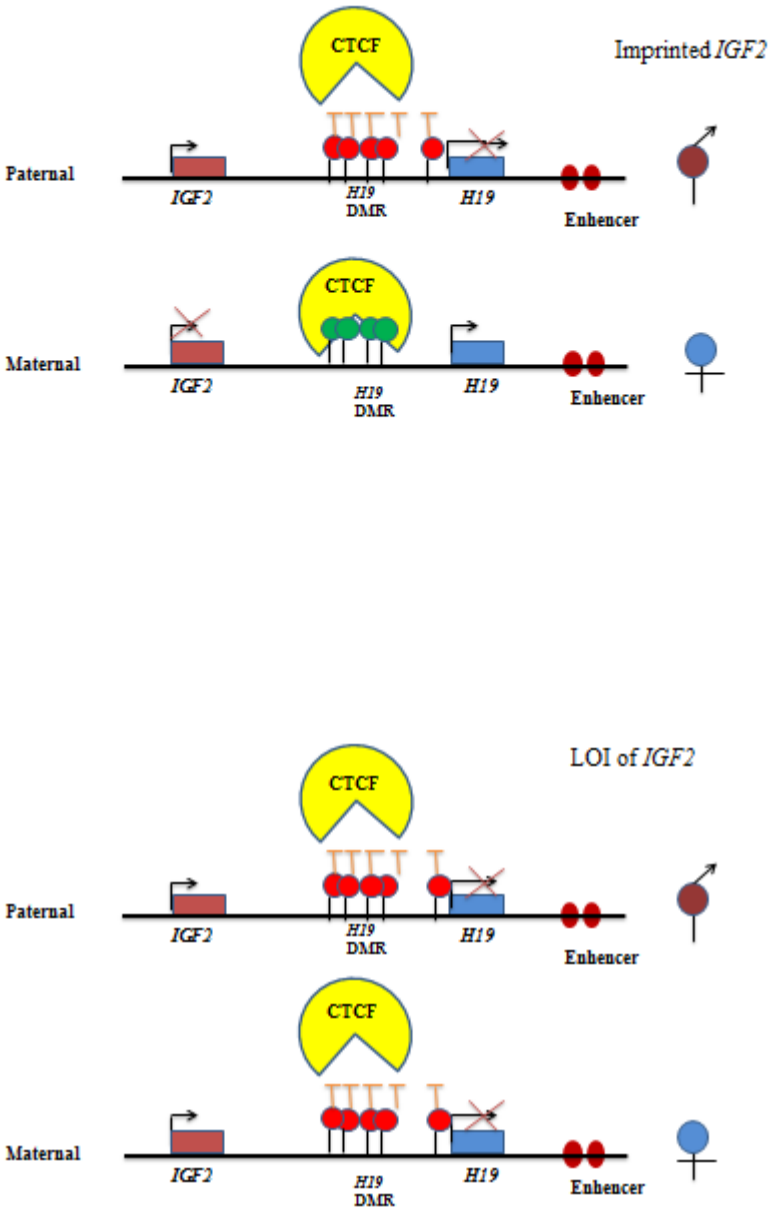

Figure 14. Mechanism of LOI of *IGF2*

1.6 PLAG1- MECHANISM IN CANCER

Pleomorphic Adenoma like gene1 or *PLAG1,* is a proto oncogene with location to long arm of human chromosome 8, (8q12)[105]. The PLAG1 protein is a zinc finger transcription factor with nuclear localization which has an important role for normal development of the salivary gland[106,107]. During different types of oncogenesis *PLAG1* becomes re-activated. Chromosomal rearrangement is the cause of re-activation of *PLAG1* and thus overexpression in different types of cancer. There are two frequent translocation, t (3:8) (p21; q12) shown in Figure 15 and t (5; 8) (p13; q12). This leads to replacement of the normally inactive *PLAG1* promoter with the active promoters of β-catenin (CTNNB1) or leukaemia inhibitory factor receptor (LIFR) genes[108]. This results in over expression of *PLAG1* in tumour cells and thus deregulation of target gene thereby causing excessive cellular growth and proliferation. One of the PLAG1 target genes is *IGF2* [107] and overexpression of *PLAG1* is associated with increased *IGF2* expression from its P3 promoter in various cancers like hepatoblastoma [109], lipoblastoma [110], and leukaemia [111], although PLAG1 was first discovered in pleomorphic adenoma of the salivary gland [112,113]. Other *PLAG1* target genes are Cytokine-like factor-1 (*CRLF1),* Bone derived growth factor (*BPGF*-1), Choriogonadotropin beta chain (*CGB),* Vascular endothelial growth factor *(VEGF)* and Placental growth factor (*PIGF)* [105].

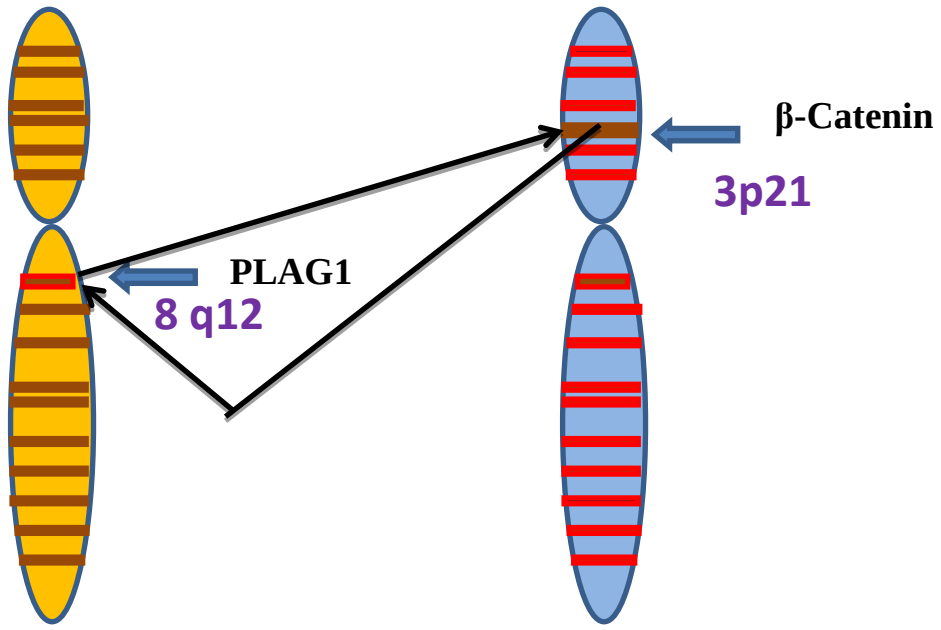

Figure 15. Translocation of the β catenin promoter with *PLAG1* promoter.

Recent studies have shown that the transcription factor PLAG1 is a main target for deregulated miRNAs in Chronic Lymphocytic Leukaemia (CLL). Pallasch *et al*, 2009 *revealed* that six miRNAs named as miR-181a, miR-181b, miR-107, miR-424, miR-155 and miR-141 are down regulated in CLL with associated *PLAG1* deregulation[114]. Predicted microRNA binding site in PLAG1 is shown in Figure 16.

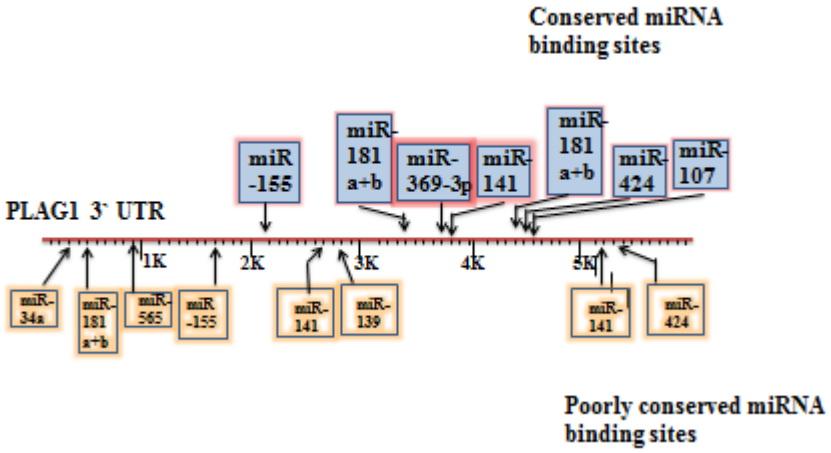

Figure 16. Schematic representation of predicted mi-RNAs binding site at PLAG1 3` untranslated region (UTR).

2 AIMS OF THE STUDIES

The general aim of the study was to investigate epigenetic mechanism and regulatory features of human cancer and neural progenitor cells which may be used for diagnosis and therapeutic opportunities.

Specific aims were:

1. To explore the effect of HDAC inhibition on intercellular gap junctional protein connexin 43 (Cx43) between neural progenitor cells (NPCs) and glioblastoma multiforme cells to facilitate NPCs as a potential vector for glioblastoma treatment.
2. To investigate epigenetic mechanisms induced by HDACi 4-phenyl butyrate on differentiation of human embryonic NPCs and its association with *de novo* DNA methyltransferase, DNMT3B.
3. To examine the role of transcription factor Pleomorphic Adenoma Like Gene 1 (PLAG1) on *IGF2* P3 promoter regulation in two different human cancer cell lines.
4. To analyse the association of the proteomic profile with transcriptional aberrations at the *H19/IGF2* locus, among two groups of adrenal tumours: adrenocortical adenoma (ACA) and adrenocortical carcinoma (ACC).

3. METHODS

3.1 Immunocytochemistry (ICC)

Immunocytochemistry is a microscopic technique which is used to identify a target protein, peptide or other antigen (e.g. nucleic acids and their modifications, lipids) in cells (either cultured or cell suspension) by using a specific antibody, which binds with the target epitope (Figure 17) and thereby become visualized under microscope after various staining techniques [115]. This is a useful tool for the detection of individual cellular contents [116]. Except cultured cells, blood smears, aspirates, and swabs can also be used. Similar analysis of tissue sections is referred to as immunohistochemistry. The experimental procedure involves sample preparation, application of primary antibody, secondary antibody, staining and visualization under microscope. Sample preparation involves fixation, permiabilization of cell membranes, blocking procedures for avoiding binding to non-specific epitopes, antibody binding and counter staining with Hoechst or DAPI. Depending on the type of cell used, the sample preparation is slightly different. The detection methods for visualization of antibody/antigen interaction can vary depending on sample type and detection limits. Direct detection (Figure 17, left) uses a single type antibody directed to the antigen in question, and can be conjugated with an enzyme such as horse radish peroxidase. During the detection phase, the sample is incubated with a solution where the peroxidase catalyzes a color reaction which can be observed under the microscope. An alternative and popular detection is to conjugate the antibody with a fluorophore, such as fluorescein or Rhoda mine for direct detection. Indirect detection (Figure 17, right), employs the same principles, but the primary antibody binding to the sample is targeted by a secondary antibody carrying the conjugated detection molecules. This method amplifies the signal substantially. In our laboratory we practice an indirect method which has been used for the first two papers in this thesis.

After the detection steps, the sample slides are mounted with a coverslip with a drop of mounting medium to seal the sample. To prevent drying and movement under microscope, coverslip can be sealed with nail polish and slides can be stored in dark at minus 20 °C or +4 °C for microscopic examination.

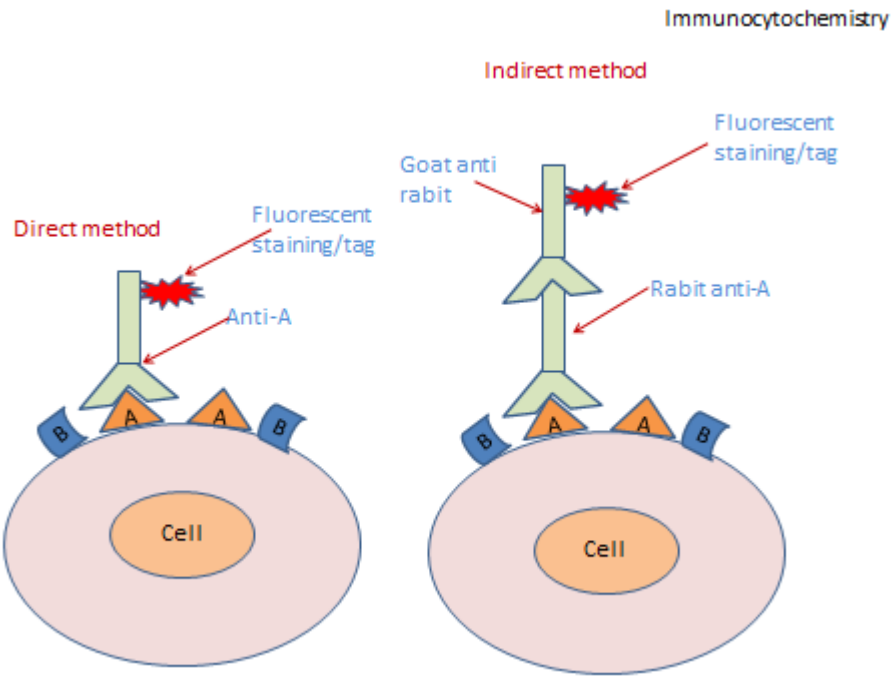

Figure 17. Illustration of the basic principles for immunocytochemistry using direct and indirect detection.

3.2 Western Blot (WB)

Western Blot, (WB) or protein immunoblotting is a widely used technique to separate and label specific proteins for identification, size estimation and quantification, from tissue homogenates or cellular extracts. The proteins are separated by sodium dodecyl sulfate (SDS), polyacrylamide gel-electrophoresis (PAGE), followed by transfer of the separated proteins from the gel onto nitrocellulose or Polyvinylidene difluoride (PVDF) membrane and then immunoblotting of the specific protein with antibodies [119]. The procedure involves sample preparation, gel electrophoresis, transfer of protein from gel to membrane, and immunostaining of the blot [120]. The method starts by lysing of the sample to release and solubilize the protein allowing migration through a separating gel. However, when the lysis begins other alterations such as proteolysis, dephosphorylation and denaturation also take place. These events can be slowed down by keeping the samples on ice or at 4 °C and by addition of enzyme

inhibitors to the lysis buffer [121]. After protein separation using PAGE, the content of the gel is transferred to the membrane. The detection procedure involves an enzyme reporter similar to immunocytochemistry. Since the membrane readily binds proteins including antibodies, a blocking procedure to minimize this unspecific binding to the membrane first needs to be performed. This is usually done with solubilized skimmed milk solutions. As is usually done in ICC, a primary antibody directed to the protein of interest is first allowed to bind, followed by an amplification (several secondary antibodies bind each primary antibody) by secondary antibody conjugated with an enzyme reporter, most commonly horse radish peroxidase which can cleave a chemo luminescent molecule in the detection solution to create light. This can be detected by film or by sensitive CCD cameras [122].

3.3 Polymerase Chain Reaction (PCR)

PCR is a widely used technique for selective amplification of DNA [123]. The method is highly efficient so that infinite copies can be made of the target sequences. The basic PCR was invented in 1983 by Kary Mullis [124] , and its many different applications have revolutionized molecular biology and medicine since it started to become widely used in the late 1980s. DNA can be directly amplified while RNA needs to be reversed transcribed to cDNA before PCR is applied. There are so many different protocols and applications resting on PCR, that it is not possible to bring them up here. However, the basic principles will be mentioned. PCR is used to amplify sequences for further use, e.g. for cloning purposes or for making DNA standards to be used in various applications. It is used for sequencing applications, for analyses of DNA single nucleotide polymorphisms (SNPs), and for quantification of nucleic acids, to mention the most obvious. In this thesis, PCR was used to identify and semi-quantify a restriction fragment length polymorphism (RFLP) in the *IGF2* gene, and for the exact quantification purposes of various mRNAs. In the first case genomic DNA and cDNA (reversed transcribed from RNA) was used followed by regular PCR and visualization of products after agarose gel electrophoreses. In the 2nd case where more exact quantification was needed, quantitative real time PCR (qPCR) was applied. For qPCR, special instruments are used where amplified products are continuously assayed during the amplification reactions.

The conventional PCR reaction includes: target DNA; two primers of approximately 20 nucleotides in length, one for each strand of the DNA; DNA polymerase; and the four nucleotides of DNA, adenine, guanine, cytosine, and thymine. For qRT-PCR, the master mix in addition also contain a DNA binding dye such as SYBR Green I or the reporter molecule which is a sequence-specific probe composed of an oligonucleotide labeled with a fluorescent dye plus a quencher (e.g. TaqMan® probes).
The qPCR reaction includes three major steps which are repeated for 30 or 40 cycles - denaturation, annealing and extension (Figure 18).

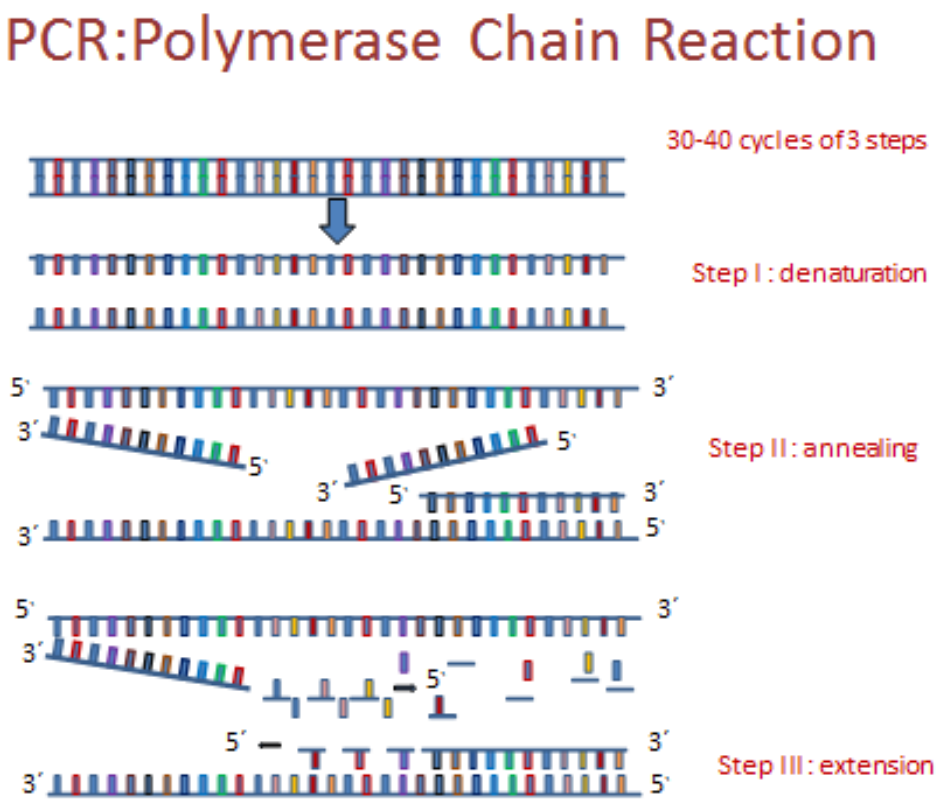

Figure 18. Schematic illustrations of the PCR method with 30-40 cycles of the three steps of denaturation, annealing and extension.

The PCR reaction is carried out for 30-40 cycles in an automated cycler machine, which can accurately change the temperature in a very short time. Denaturation is carried out at 94 °C to generate single stranded DNA. During annealing the primer anneals or hybridizes itself to its target on the 5´ side of the DNA to be amplified on one of the strands. DNA polymerase then attaches to a primer template to extend the

primer. Polymerization starts when the reaction extents to correct temperature [123], whereby the enzyme adds nucleotides to a growing strand of DNA. Since both DNA strands are copied, the number of target copies will increase exponentially. If we start from one target copy, there will be two copies after the first cycle, followed by 4, 8, 16 and so on[125]. This exponential phase is followed by a plateau phase due to consumption of the reaction components [126] (Figure19).

The PCR result is analyzed in different ways depending on the application. Commonly the software provided with the real-time PCR instrument is used. In conventional PCR, the amplified products visualized by agarose gel electrophoresis. In real-time PCR the amplification product is measured during the reaction, i.e. in real time, whereby the product is quantified after each cycle. As illustrated in Figure 19, the number of cycles is plotted against the fluorescence , which is proportional to the amount of amplified product.[126]

PCR is used to analyze DNA and gene expression and has applications for basic scientific as well as medical questions. Studies of evolution, human disease, identification of criminals are some examples of fields relying on PCR. PCR can be applied to study DNA from all sources, such as detection of HIV in human blood samples.

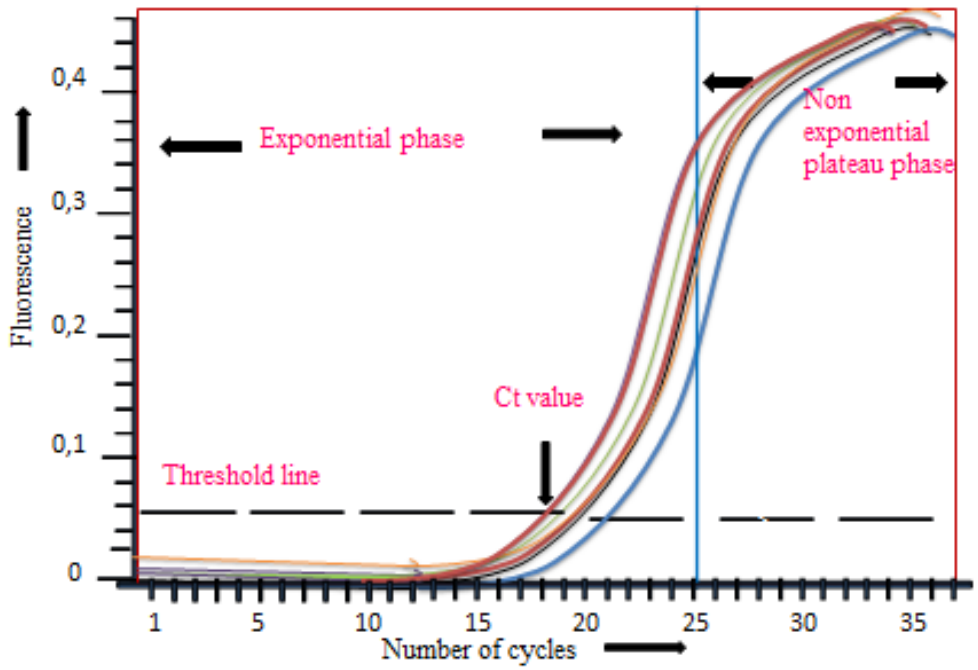

Figure 19. Amplification by real-.time PCR. The number of PCR cycles is shown on the x-axis, and the fluorescence on the Y-axis. The exponential phase and the non-exponential plateau phase are shown. The Ct value refers to number of cycle at which amplification plot crosses the thresh hold line and the threshold line is a horizontal line drawn at the amplification plot. When threshold line is crossed by amplification plot then it indicates ct value.

3.4 Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is used to determine whether a specific protein binds to or is localized to a specific DNA sequence[127-129]. The mains steps of the method are outlined in Figure 20. ChIP has been widely used for studying chromatin remodeling, mapping the position of modified histones, transcription factors, and other nonhistone proteins in the genome. In a conventional ChIP, DNA and proteins are reversibly cross-linked to maintain the association of proteins with target DNA sequences. The chromatin is then sheared by sonication to achieve fragments of 200 ~500 bp. Large complexes are removed by centrifugation, and the supernatant is used for immunoprecipitation. In native ChIP (nChIP), which is e.g. used for mapping histones to specific sequences, crosslinking is omitted and the natural binding between histones and DNA is exploited [129]. Instead of sonication, micrococcal nuclease is used

to cut the DNA in between the histone containing nucleosomes. A specific antibody conjugated with e.g. a biotin is then used to bind the protein-DNA complexes. Streptavidin conjugated magnetic beads, or columns are then used to capture the biotin-protein complexes. The immune precipitated complexes are washed, cross linking (if used) is chemically reversed, proteins are digested and the DNA that is left is collected. The DNA can be directly quantified by qRT-PCR or stored at -20 ^{0}C for up to 1 to 2 weeks [127]. The captured sequences can be amplified by qRT-PCR (ChIP-PCR) [128], cloning and high-throughput sequencing (ChIP sequencing) [130] or, hybridization to microarray (ChIP-chip) [131]. For ChIP-PCR used in this thesis, all ChIP samples are run in triplicate and then mean quantities are used for all subsequent analysis.

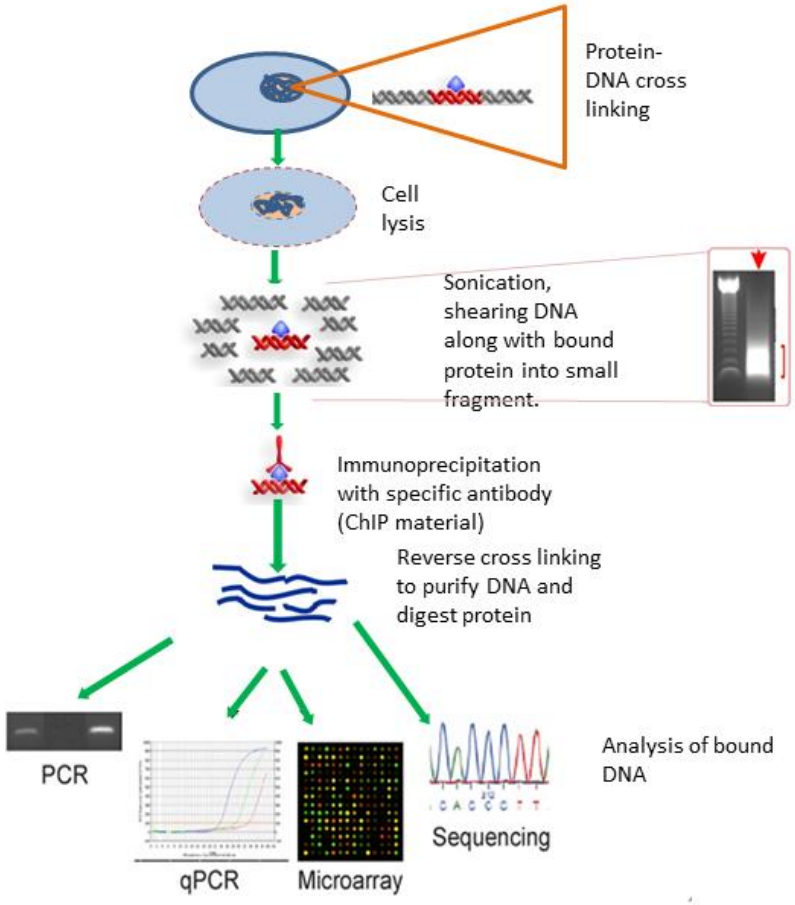

Figure 20. The main steps of the ChIP procedure.

3.5 LUMINOMETRIC METHYLATION ASSAY (LUMA)

LUMA is a global DNA methylation analysis method that is based on the use of methylation sensitive and resistant restriction enzymes, and quantification by Pyrosequencing of amount of cleavage in the genome in relation to an internal standard. LUMA uses two isoschizomer enzymes for the sequence CCGG, *Hpa*II or *Msp*I, to cut genomic DNA. *Hpa*II will only cut if the internal cytosine is un-methylated whereas *Msp*I will both methylated and un-methylated DNA. Both leave a 5´ overhang. The *Eco*RI restriction enzyme is used in all reactions to normalize between the *Hpa*II and *Msp*I runs. The amount of restriction cleavage is then quantified by Luminometric polymerase extension assay where the overhangs created are filled in while producing a peak of light which is proportional to the number of cuts. DNA methylation is expressed as the relative ratio of *Hpa*II / *Msp*I normalized to *Eco*RI. Theoretically, the ratio should be 1.0 if DNA is completely unmethylated, and should approach 0 when it is completely methylated.

The basic phases of the LUMA technique and the nucleotide dispensations are illustrated in Figure 21.

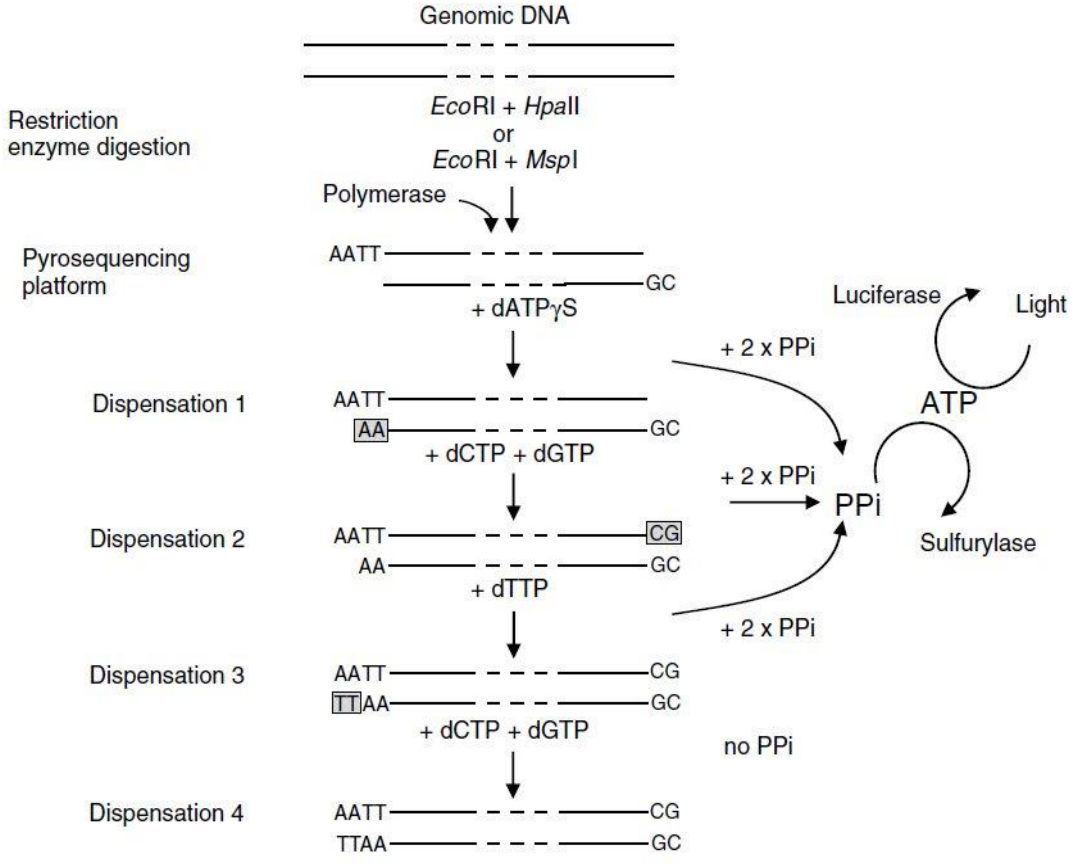

Figure 21. Illustration of the LUMA method.

An example of how to analyze the data from LUMA is shown in Figure 22. All overhang generated by *Eco*RI is completed by addition of first dATPαS which provides A peaks and then dTTP which create the T peak. The C+G peak represents ratio of cuts filled by *Msp*I or *Hpa*II. The final C+G peak represents any non-complete filling-in, and can typically be seen if the analyzed DNA was degraded. In the case shown the methylation in CCGG sequences is determined to be 57% (1-0.9/2.1).

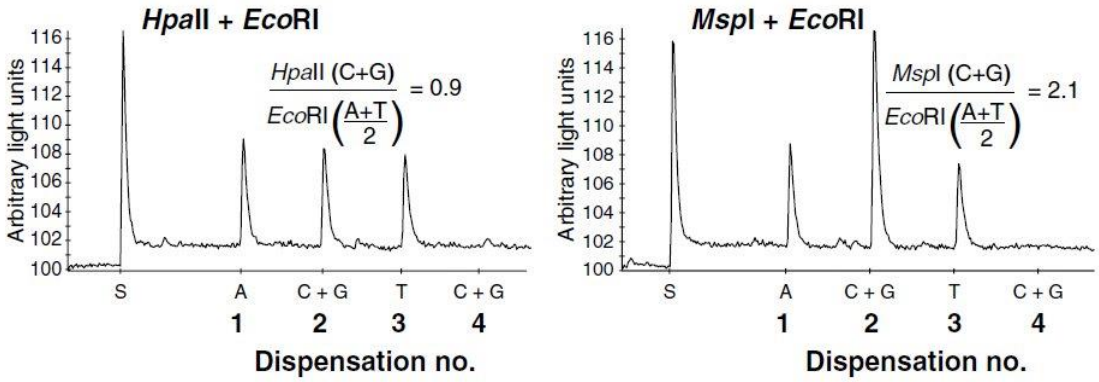

Figure 22. Example of results from LUMA. From Karimi *et al*, 2006; Epigenetics

3.6 DETECTION OF GENOMIC IMPRINTING

Genomic imprinting means that a specific allele is marked in a parent of origin fashion. Functional genomic imprinting means that this mark has consequences for gene expression in allele-specific way[132,133]. This can be analyzed and quantified. Loss of genomic imprinting (LOI) is an event where the normally silenced allele is reexpressed together with the normally active allele. Thus a mono allelic to biallelic expression of a gene occurs. Insulin-like Growth Factor 2 (*IGF2*) is a maternally imprinted gene and LOI of *IGF2* has been shown in many different cancers. Due to activation of the normally silent maternal allele both alleles of *IGF2* are expressed which theoretically would double the gene dosage[100].

The principle for analyzing allele-specific expression is to exploit natural restriction length polymorphisms (RFLPs) generated by single nucleotide polymorphisms (SNPs) in restrictions sites present in gene exons. In this way, parental origins can be

determined if the parents' DNA is available and if paternal and maternal DNA is homozygous for different SNPs. If RFLPs exist, simple cutting of a PCR amplified product spanning the cutting site may be employed, followed by gel electrophoresis to separate the generated differentially sized bands. If the parents' alleles are different, an offspring's parental alleles can be traced back. It is thus necessary that in an individual' both alleles carry different polymorphisms at a specific site in order to distinguish them. To analyze the expressed alleles, cDNA is generated and cut with the same enzymes. If only one allele is expressed, only the cut or uncut band will be present, and if the parental type of allele is known it may be possible to determine which parental alleles is being expressed.

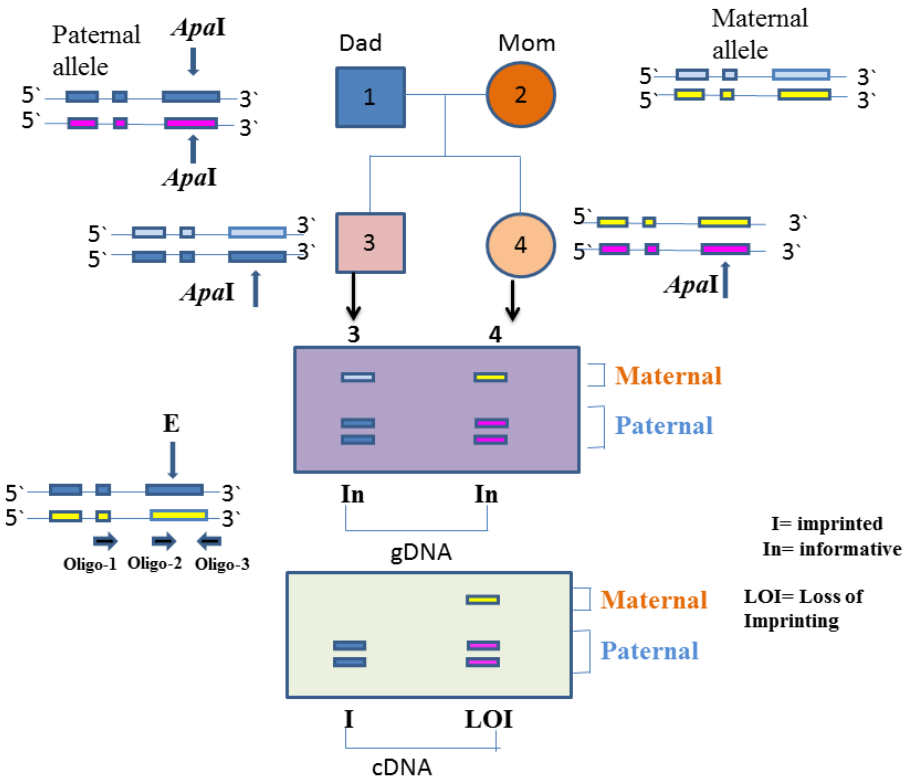

Figure 23. Detection of loss of imprinting (LOI). In this example paternal allele contains a single nucleotide polymorphism (SNP) that is recognized by *Apa*I restriction enzyme but maternal allele lacks this site (but in fact any allele can contain SNP). Oligonucleotide primers Oligo-2 and Oligo-3 can only amplify genomic DNA (gDNA) while Oligo-1 and Oligo-3 can only amplify cDNA or RNA. In example 3 & 4 gDNA shows both paternal and maternal allele marked as informative (In). These informative samples are run for RT-PCR with cDNA and digest with *Apa*I or *Eco*R1 which can detect only allele that contains *Apa*I restriction site. In this example expressed allele is paternal and silent allele is maternal.

In most clinical cases however, the parental origin of a particular allele type is not known. Nevertheless, it may still be possible to determine mono- or bi-allelic expression if the individual is heterozygous for the RFLP [134]. For *IGF2***,** an *Apa*I RFLP in exon 9 is commonly used to distinguish alleles[100]. If genomic DNA analysis shows that both allele types are present, those are considered as informative samples which can be used for analysis of cDNA. The informative samples are run for specific RT-PCR amplification of a cDNA template. The primer for the RT-PCR is usually placed in different exons, so that it is possible to distinguish cDNA from contaminating genomic DNA. Digestion of the RT-PCR product with *Apa*I restriction enzyme followed by agarose gel electrophoreses can then detect cut and uncut transcripts. In the case of monoallelic expression, the cut or uncut band only, will be present. In the case of biallelic expression, both the cut and uncut bands will be shown on the gel. The method is outlined in Figure 23.

4. RESULTS AND DISCUSSION

4.1
Paper I
HDAC inhibition amplifies gap junction communication in neural progenitors: Potential for cell-mediated enzyme prodrug therapy.

The purpose of this study was to investigate the effect of HDAC inhibitor, 4-PB on inter-cellular communication through the gap junctional protein connexin 43 (Cx43) between neural progenitor cells (NPCs) and glioblastoma multiforme cells. Cx43 is an intercellular junctional protein which may facilitate transfer of therapeutic molecules between cells. This will enhance the employment of NPCs as a potential delivery vehicle for glioblastoma therapy.

For this purpose we have used two NPC cell lines, NGC-470 and HNSC.100, both of human embryonic origin and with neural precursor characteristics, and two established Glioblastoma multiforme (GBM) cell lines, U87MG and U343MGa. The NGC-470 cell line was established by immortalization from ventral mesencephalon of a 7-week-old human embryo by using a retroviral vector containing the v-myc oncogene. The human HNSC.100, embryonic forebrain-derived, was used as a reference cell line.

Differentiation of NGC-407 cells

Cells were grown with and without basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) which promoted proliferation and differentiation by the presence or absence of the mitogens, respectively. Almost all cells were strongly positive for the neural stem cell marker nestin, while <5% stained for the astrocytic marker GFAP and <1% was positive for the neuronal cell marker β-tubulin III. After 5 days of differentiation, the majority of cells differentiated into astrocytes with GFAP expression. Nearly 10% of cells changed into neurons with positive staining for the neuronal marker β-tubulin III.

Modulation of Cx43 expression by the HDAC inhibitor 4 phenyl butyrate, 4-PB

ICC demonstrated increased Cx43 immunostaining both in intensity and peripheral distribution after adding 4-PB to cultures to proliferating and differentiating cells, with additional Cx43 expression in differentiating 4-PB treated cells in comparison to untreated cultures. Cx43 in differentiated cells was distributed along the plasma membrane of cell surface whereas proliferating cells showed highly polarized distribution independent of cell density with areas of patchy fluorescence in the cytoplasm as well as granular appearance on the cell surface. WB analysis of whole cell extracts distinguished three immunoreactive forms of Cx43, smaller non-phosphorylated, larger phosphorylated and hyper phosphorylated (or total) form. After 4-PB treatment, proliferating NGC- 407cells showed an increase in total Cx43 and a decrease in Ser 279/282-phosphorylated. However, total Cx43 in differentiating cells was lower both before and after treatment with 4-PB, in comparison to proliferating cells, although 4-PB increased both the total and Ser 368 phosphorylated forms. Interestingly, 4-PB changed the relative distribution of the two different phosphorylated forms to total Cx43 in different ways in proliferating and differentiating cells. In proliferating cells, the relative proportion of both phosphorylated forms decreased significantly, while in differentiating cells, only the Ser 279/282-phosphorylated form decreased. This may have functional consequences for gap junction communication.

Functional gap junctional communication

NGC-407 cells were labeled with two fluorescent probes, lipophilic membrane-bound and non-transferable, Dil and a water soluble acetomethylic ester, Calcein, which can diffuse via gap junctions to neighboring cells. Double labeled NGC-407 cells were co-cultured with unlabeled NGC-407 or U343MGa or, U87MG cells under two different conditions, with and without 4-PB pre-treatment. After 4 hours, 4-PB pre-treated proliferating NGC-407 showed a substantial increase in intercellular dye transfer into both U343MGa and U87 cells in comparison to untreated cells. This was evident under both proliferating and differentiating conditions.

We conclude that HDAC inhibitor 4-PB increases gap junctional protein Cx43 in in-vitro model and that the neural progenitor cell paradigm is promising for use as a delivery vehicle for enzyme prodrug therapy for glioblastoma treatment.

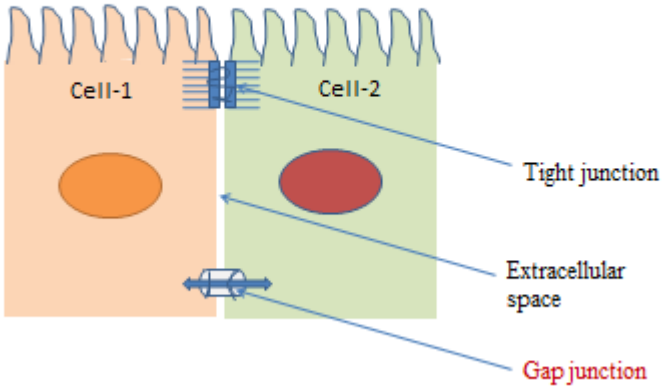

Figure 24. Figure showing gap junction

4.2
Paper II
HDAC inhibitor 4-phenylbutyrate preserves immature phenotype of human embryonic midbrain stem cells: Implications for the involvement of DNA methyltransferase.

The purpose of this study was to explore the role of *de novo* DNA methyltransferase DNMT3B for neural stem cell differentiation and the effect of the HDAC inhibitor 4-PB.

For this purpose we used the human embryonic midbrain stem cells, NGC-407, which displays increased expression of DNMT3B in differentiating cells in comparison to proliferating cells. When these cells were treated with 4-PB, differentiation of both astrocytic and neuronal form were suppressed along with reduction in DNMT3B immunoreactivity in the cell nuclei.

Effect of 4-PB on neuronal stem cell marker by Immunocytochemistry (ICC) and Western Blot (WB)

ICC showed that 4-PB treated NGC-407 cells under differentiating conditions preserve their neural stem cell marker, nestin, while no considerable change was seen in 4-PB treated proliferating cells. On the other hand, WB realized the presence of a short and long form of nestin in proliferating cells both before and after 4-PB treatment. On the other hand, 4-PB treated differentiating cells retained the long form of the neuronal marker while the short form degraded after 5days of differentiation. CD133, another stem cell marker, also showed significant up regulation in 4-PB treated differentiating cells in comparison to untreated.

Effect of 4-PB on astrocytic and neuronal differentiation marker

When NGC-407 cells are allowed to differentiate by removal of mitogen, astrocytic (GFAP) and neuronal (β-tubulin) markers are unregulated. However, simultaneous treatment with 4-PB, prevented the appearance of both these proteins indicating that the HDAC inhibitor inhibited both astrocytic and neuronal differentiation. This was also supported by the lack of development of cell processes and fine networks.

Global DNA methylation assessment in NGC-407 cells by LUMA

Differentiating NGC-407 cells showed increased global DNA methylation in comparison to the levels in proliferating cells. Upon treatment with 4-PB, global DNA methylation level was somewhat, but not statistically significant decreased in both proliferating and differentiating cells in comparison to untreated cells.

Outcome of HDAC inhibitor 4-PB on DNA methyltransferase

Proliferating untreated NGC-407 cells showed both long and short isoform of the maintenance DNA methyltransferase, DNMT1, by WB analysis. 4-PB substantially reduced the longer form while no change was seen in the shorter. However, upon differentiation the longer form completely disappeared while the shorter form increased. Treatment of the cells with 4-PB did not significantly alter this situation. The suggested function of DNMT1 is to maintain methylation through cell division. It appears that the longer form of DNMT1 is important for this function since it so prevalent in the proliferating cells, while the shorter form, abundant in differentiated cells, may not display the same mode of action. This finding was also supported by ICC which showed a more specific type of nuclear staining of DNMT1 in proliferating cells compared to differentiating cells. When cells were treated with 4-PB, DNMT1 immunoreactivity showed a diverse pattern, even outside the nucleus in proliferating cells, without any obvious effect in differentiating cells.

WB investigation did not show any change of DNMT3A protein level between proliferating and differentiating cells. However, differentiating cells showed increased level of DNMT3B compared to proliferating cells. 4-PB treated proliferating cells showed slight down regulation of DNMT3A but no change of DNMT3B as compared to differentiating cells. ICC demonstrated both perinuclear and cytoplasmic localization of DNMT3A with nuclear restriction for DNMT3B. In comparison to untreated 4-PB cells, immunoreactivity for both DNMT3A and DNMT3B was markedly reduced in proliferating and differentiating 4-PB treated cells.

The result of this study supports the involvement of epigenetic events, like association of HDACs on human embryonic NGCs differentiation. Epigenetic mechanisms behind the effect of HDAC inhibitor, 4-PB may involve *de novo* methylation by DNMT3B, leading to prevention of astrocytic and neuronal differentiation, and thereby preservation of the immature phenotype.

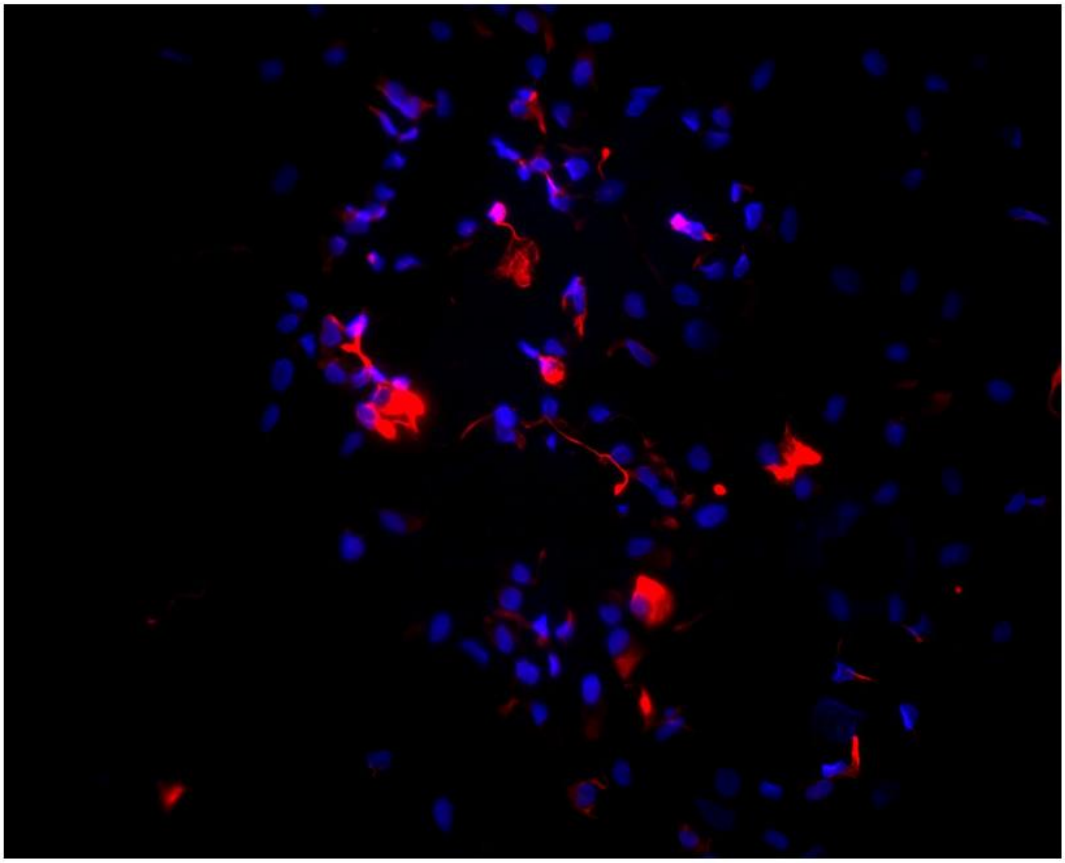

Figure 25. Immunocytochemistry showing cells positive for nestin.

4·3
Paper III
Cell type and context-specific function of PLAG1 for *IGF2* P3 promoter activity.

The purpose of this study was to investigate the role of the transfection factor Pleomorphic Adenoma Like Gene 1 (PLAG1) on regulation of *IGF2* P3 promoter activity in two different cancer cell lines. We also examined if PLAG1 can partially override the insulation of *H19* ICR in cancer cells using a green fluorescent protein (GFP) reporter construct.

Cell line and stable cell clones

Hep3B and JEG-3 cell lines and a zinc inducible *PLAG1* expressing stable clone from JEG-3 cells were used for this study.

Quantitative real-time PCR (qRT-PCR) analysis of *IGF2* and *PLAG1*

We examined the endogenous level of *IGF2* and *PLAG1* expression in Hep3B and JEG-3 cells by qRT-PCR. Hep3B cells showed higher level of *IGF2* (around 6-fold) and *PLAG1 (*almost 3-fold, but both at a very low level) expression in compare to JEG-3 cells, by using GAPDH as the normalizing gene. After transient transfection with *PLAG1* expression vector, *IGF2* and *PLAG1* expression was analyzed in these two cell lines. Although Hep3B cells showed 2,000-fold induction of *PLAG1* mRNA only a modest increase was found in *IGF2* expression. The JEG-3 cells showed 130-fold up-regulation of *PLAG1* mRNA (from a very low level) but without any effect on *IGF2* expression.

Chromatin Immunoprecipitation (ChIP) analysis

To investigate the binding of PLAG1 to the *IGF2* P3 promoter, we performed ChIP in Hep3B and JEG-3 cells. For this purpose cells were transiently transfected with a *PLAG1* expression plasmid. Transiently transfected Hep3B cells showed increased binding of PLAG1 to *IGF2* P3 promoter compared to control non transfected cells. JEG-3 cells however, showed no PLAG1 binding, neither in non-transfected cells, nor

in transfected cells. The additional expression of *PLAG1* by transient transfection did not increase the endogenous level of *IGF2* in JEG-3 cells.

IGF2 P3 promoter driven reporter constructs expression in the presence of a functional insulator

The ChIP assay showed that increased PLAG1 expression did not result in binding to the P3 promoter in JEG-3 cells, but did so in Hep3B cells. Furthermore, *IGF2* expression was not elevated in JEG-3 cells although their WT expression was very low. We therefore hypothesized that Hep3B and JEG-3 cells might have different sensitivity to the important *H19* upstream insulator and that PLAG1 might be involved in this regulation. In order to get a clue, we started to investigate the insulator effect of the ICR in these two cell lines. We used an *IGF2* P3 promoter driven GFP based reporter constructs containing the ICR with or without a SV40 enhancer. These constructs were first tested in both Hep3B and JEG-3 cells together with a RSV-driven red fluorescent protein (RFP) vector as a control, using both confocal microscopy as well as FACS sorting. It was evident that the Hep3B cells were insensitive to the insulator in contrast to the JEG-3 cells. We therefore analyzed the insulator effect in JEG-3 cells with a Zn-inducible PLAG1 expression vector, using both confocal microscopy as well as FACS sorting to find out whether the ICR could be overcome by PLAG1. The outcomes of these experiments suggest that the insulator function in the construct is partially overridden by PLAG1 expression in this system.

Since the ICR displayed different effects in the GFP-constructs depending on cell line, the GFP reporter constructs, driven by the *IGF2* P3 promoter and containing the *H19* imprinting control region (ICR) insulator was transfected into both cell lines chromatin conformation assays of the ICR was conducted using DNase I hypersensitivity. The results suggest that the chromatin conformation of the transfected ICR was similar in both Hep3B and JEG-3 cell line.

PLAG1 expression attenuates the insulator function of the *H19* ICR

In order to answer the question if *PLAG1* over expression in JEG-3 cells could evoke a similar effect as in Hep3B, we generated a stable zinc-inducible *PLAG1* clone in JEG-3 cells. These cells were co-transfected with the *GFP* and *RFP* (control) constructs and analyzed by qRT-PCR. Although the enhancer containing, insulator-less construct pA-GFP showed substantially higher GFP expression after 48 hours Zn induction in

absolute numbers, the insulator-containing construct increased a similar-fold by Zn-induced PLAG1 expression. This suggests that the insulator function is lost to some extent in JEG-3 cells by *PLAG1* overexpression.

Finally, we suggest that the PLAG1 binding to the *IGF2* P3 promoter and *IGF2* expression is cell type-specific, and that the PLAG1 transcription factor acts as a transcriptional facilitator that partially overrides the insulation by the *H19* ICR in some cell contexts.

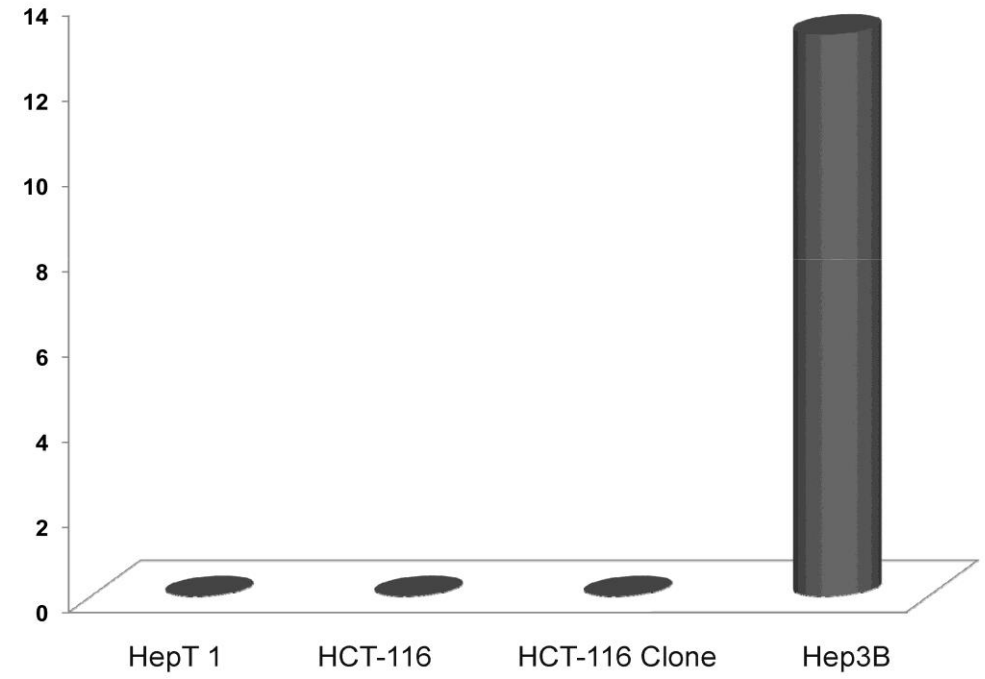

Figure 26. ChIP result showing PLAG1 binding at *H19* ICR

4.4
Paper IV
Proteome profiles associated with alterations of the *IGF2/H19* locus and malignancy in adrenocortical tumors.

Over-expression of the *IGF2* gene is a very frequent alteration in adrenocortical carcinoma (ACC) but not in adrenocortical adenoma (ACA). At the same time the functional evidences for a major tumor driving role of *IGF2* are weak in ACC and in other tumors where *IGF2* over-expression is frequent[135,136]. The *IGF2* gene is situated in the imprinted *IGF2/H19* locus from which additional molecules are generated that may each potentially contribute to ACC development. These include *miR-483-3p* and *miR-483-5p* transcribed from *IGF2*, *H19* and *miR-675* transcribed from *H19*, and *HOTS*. The purpose of this study was to explore aberrations at *H19/IGF2* locus in ACC and relate this to protein expression profiles determined by proteomics and reported elsewhere (Eriksson et al. in manuscript). A screening series of 5 normal adrenal cortices, 6 ACAs and 8 ACCs was first used. An extended series of 9 normal adrenal cortices, 43 samples of ACAs and 30 samples of ACCs were then used for verification of findings by qRT-PCR.

Expression of *IGF2*, *H19*, *HOTS* and *PLAG1*

Using qRT-PCR in the screening series we found that *IGF2* was increased in ACCs compared to ACA and control samples. Elevated levels of *H19* were observed in almost all ACAs, while all ACCs showed down regulation of *H19*. We also included *HOTS* and *PLAG1* expression at the mRNA level in our study group. All samples showed homogenous expression of *HOTS* and *PLAG1* mRNA without clear discrepancy between the ACA and ACC. This result suggested that *PLAG1* and *HOTS* are not important for the development of ACC. Hence, HOTS and PLAG1 were excluded from further investigation.

Expression of *miR-483-3P*, *miR-483-5P* and *miR-675*.

miRNA generated from the *H19/IGF2* locus are *miR-483-3P*, *miR-483-5P* and *miR-675*. *miR-483-3P* and *miR-483-5P* created from the *IGF2* locus were up regulated in ACCs compared to ACAs and control, whereas *miR-675* produced from the *H19* locus

was down regulated in ACCs in relation to ACAs. Because of diverse expression between the two groups of tumors, we further analyzed these transcripts in an extended series of samples. *miR-483-3P*, *miR-483-5P* and *IGF2* mRNAs were up-regulated in ACCs compared to ACAs and control, whereas *H19* mRNAs and *miR-675* showed down-regulation in the ACC group. This association between mRNAs and miRNAs investigated in the extended series disclosed a weak inverse correlation between *IGF2* and *H19*. Positive correlations were observed between *IGF2* and *miR-483-3P* and *miR-483-5P*, as well as between *H19* and *miR-675*.

Infrequent loss of imprinting in ACC

To assess the allelic expression of the imprinted *IGF2*, an *Apa*I polymorphism in exon 9 of the *IGF2* gene was used in our screening of genomic DNA from the 8 ACCs and 6 ACAs. We identified in total eight informative samples based on restriction fragment length polymorphism, RFLP, which we perceived after *Apa*I digestion, i.e. heterozygosity for the *Apa*I site. These eight informative samples were further analyzed for detection of loss of functional imprinting (LOI) of the silenced allele. Two samples of the eight showed LOI of *IGF2*, of which one showed complete LOI whereas the other was indicative of partial LOI. The cases with LOI were both in the ACCs group, and LOI did not correlate with expression of *HOTS* or *PLAG1*. The sample that showed complete LOI exhibited higher expression of *IGF2* and the IGFII protein.

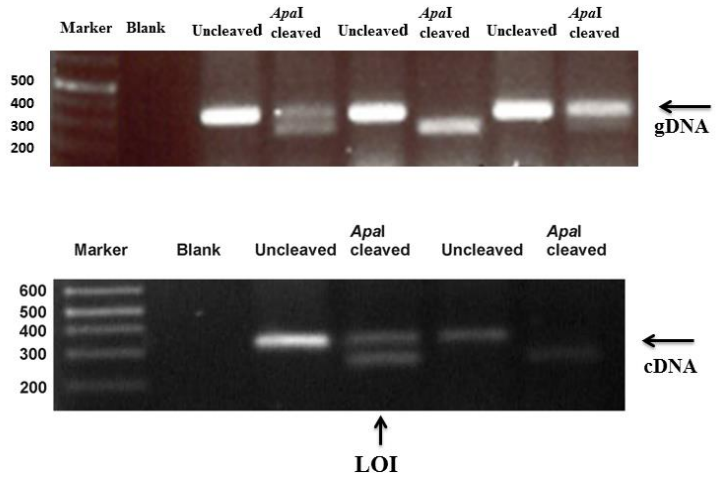

Figure 27. Informative samples and samples with LOI.

Comparison of transcripts and protein expression profiles

The proteomic analysis revealed a good correlation between *IGF2* mRNA and IGFII protein levels. ACCs which showed higher *IGF2* mRNAs also showed increased protein expression. The ACAs displayed low *IGF2* and also low IGFII. The relationship of IGF II protein with *IGF2* mRNA demonstrated a positive correlation between (r = 0.52; P = 0.055).

Mass spectrometry data obtained for the screening series of eight ACCs and six ACAs was employed. We compared these proteomic data with our mRNAs and miRNAs. With good significance, six proteins were correlated to *IGF2* mRNA and *miR-483-3P*, 48 proteins correlated to *IGF2* mRNA and *miR-483-5P*. 120 proteins overlapped for the *IGF2* mRNA, *miR-483-3P* and *miR-483-5P* correlations. Only four proteins correlated with *H19* and *miR-67*. Association of the proteomic data with *IGF2* and *H19* expression revealed 222 proteins correlating with *IGF2* and 71 proteins correlating to *H19*. We also identified proteins that inversely correlate with these three transcripts. By this we found seven proteins which inversely correlate to *miR-483-3P* expression among which two were predicted targets using three prediction programs from miRecords. Including 20 predicted targets, we found 101 proteins that correlated inversely with *miR-483-5P*. Eleven inversely correlated proteins to *miR-67* were identified.

***IGF2* and *H19* gene networks**

To assess the theoretical interactions and cellular networks of *IGF2* and *H19* correlating proteins, we used the Ingenuity Pathway Analysis "omics" data (IPA version 14400082; Ingenuity® System, www.ingenuity.com). Top networks for *IGF2* were composed of 34 molecules which implicate RNA post-transcriptional modification, expression of gene and molecular transport. The *H19* top network correlated proteins characterizes 18 molecules associated with lipid metabolism, molecular transport and small molecular biochemistry.

In conclusion, *IGF2*/*H19* locus deregulation implicates up-regulation of *IGF2* at mRNA and protein levels, along with *miR-483-3P* and *miR-483-5P* produced from this locus, as well as down regulation of *H19* with its *miR-675*. Overexpressed protein and miRNAs identified at the *IGF2*/*H19* locus in ACCs in comparison to ACAs may be established as therapeutic marker and for follow-up of patients.

5. CONCLUDING REMARKS

The development of cancer is a highly complex process, in which genetic and epigenetic alterations may contribute alone or in combination. While the field of cancer genetics is since long well-established, cancer epigenetics is a relatively younger but expanding research area. This thesis is focused on epigenetic modifications in cancer from both a descriptive and potentially therapeutic perspective. Epigenetics may be defined as heritable modifications of the chromatin that do not include changes in the DNA sequence or the proteins connected to DNA. The epigenetic modifications known today include DNA methylation, histone modification and RNA induced silencing. The findings in this thesis will help to better understand epigenetic aspects of altered methylation and histone modification in neural progenitor cells, for employment as therapy vector; the role of the transcription factor PLAG1 in regulation of the imprinted gene *IGF2*; and alterations of the imprinted *IGF2*/*H19* locus in adrenocortical carcinoma. The results can be summarized as follows:

1. We wanted to explore how neural progenitor cells (NPCs) could be used as vectors for carrying a suicide gene. The important gap junction communication between the vector cells and the target cancer cells was studied and attempts to stimulate it with epigenetic drugs were tested. Our result provides better understanding about the effect of HDACi on the gap junctional protein connexin 43 between NPCs and glioblastoma multiforme cells and we propose that HDACi treated NPCs may act as a potential vector for glioblastoma treatment.

2. In continuation of the first paper, it is important to understand how HDACi affect the differentiation potential of the attempted suicide gene vector cells, since differentiation is negatively correlated with cell migration and cancer cell tropism. We showed that HDACi prevented differentiation, possibly through involvement of DNMT3A. This emphasizes the usefulness of HDACi as an inducer of gap junction communication and NPC migration.

3. The importance of PLAG1 as a facilitator of *IGF2* transcription was studied. We found that PLAG1 display cell type-specific binding efficiency to the *IGF2* P3 promoter and that it appears to partially overcome the *H19*

insulator in a construct containing an imprinting control region. The transcription factor PLAG1 may therefore have a cell type-specific role on regulation of *IGF2* P3 promoter activity.

4. Abnormalities at the *H19/IGF2* locus in adrenocortical carcinoma include over-expression of *IGF2* and *miR-483* under-expression of *H19* and *miR-675*. The alterations are associated with altered protein expression profiles including potential diagnostic markers.

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